GENETIC DIVERSITY, DEVELOPMENT OF IMPROVED DIAGNOSTIC ASSAYS AND EVIDENCE TOWARDS THE TARO PLANTHOPPER (TAROPHAGUS PROSERPINA) AS THE VECTOR FOR TARO VEIN CHLOROSIS VIRUS (TAVCV) IN HAWAII

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DEDICATION

I dedicate this thesis, firstly, to the special people who cherished my achievements as their own and of the family; those whom now I cannot meet in person, ever, but treasure in my memories and occasionally meet in my dreams – my late paternal grandmother Satya Wati, my late father Dinesh Chandra Gosai, my late uncles Rakesh Chandra Gosai and Kamal Kumar and my late maternal grandfather Hari Prasad.

I dedicate this thesis, next, to the people who I am fortunate enough to celebrate this journey of life with. People, who beyond the struggles, the qualifications and the end result mean the world to me. To *la familia en* Fiji, U. S. A., Australia, New Zealand, Canada and Samoa – this is for you!

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ABSTRACT

Taro vein chlorosis virus (TaVCV; genus *Nucleorhabdovirus*, family *Rhabdoviridae*) is a recent discovery in Hawaii and causes veinal chlorosis with a netted appearance, stunting and petiole streaking in taro (Colocasia esculenta). Plant death may occur in severe infections. Nucleotide and amino acid sequence comparisons and phylogenetic analyses revealed extremely low levels of genetic diversity in the partial RNA-dependent RNA polymerase (RdRp) gene of 43 Hawaiian and 3 Palauan TaVCV isolates. This sequence information was used to design six new primer pairs targeting different regions of the RdRp gene. Primer set DCGF5/DCGR5 was identified as the most efficient of the six. Following optimization, highly sensitive and robust reverse transcription-polymerase chain reaction (RT-PCR) and immunocapture-RT-PCR (IC-RT-PCR) assays were developed. Localization of TaVCV in insect body parts essential for propagative, circulative virus transmission suggest that the taro planthopper, *Tarophagus proserpina*, is a vector of TaVCV.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Taro, *Colocasia esculenta* (L.) Schott

1.0 Botany and Ecology

1.1 Classification

Taro belongs to the genus *Colocasia* in the Arum family, Araceae. In this family of monocotyledonous flowering plants, close to 100 genera and 1,500 species are recognized (Whitney, Bowers, & Takahashi, 1939). Thousands of years of cultivation in different regions of the world has led to the development of hundreds of varieties and agronomical cultivars. As a result, there is confusion and disagreement amongst botanists for systematic taxonomy of the genus *Colocasia* (Onwueme, 1999).

Hill (1939) provided a comprehensive description of the efforts of several botanists to classify taro: Linnaeus in 1753 described taro of two types; *Arum colocasia* and *A. esculentum*. Schott in 1832 created the genus *Colocasia* and moved the above two species as *Colocasia antiquorum* and *C. esculenta* respectively. Schott also added a third species *C. acris*, earlier described as *Calladium acre* by Robert Brown in 1810. Kunth added a fourth species, *C. nymphaeifolia* in 1841 and Schott incorporated a fifth, *C. fontanesii* in 1854. In that same year, C. Koch and Sello reported a new species – *C. euchlora* which increased the number of species in the genus *Colocasia* to six.

Then, in 1856 Schott re-organized the classification and recognized only one species – *C. antiquorum* while the other five became ranked as varieties of this species. In 1879, Engler described two additional varieties; 'typica' and 'illustris'. Engler and Krause then added varieties 'aquatilis' and 'globulifera' in 1920. *International Rules of Botanical Nomenclature* otherwise

known as the 'Vienna Rules' of 1906, later revised to *International Code of Botanical Nomenclature* – 'Stockholm Code' in 1952 rendered the changes made by Schott in 1856 as unlawful; according to one of the rules established by the consortium, an older species could not be reduced to a varietal rank under a new species published at a later date. Consequently, literature thereafter recognize *C. esculenta* as the main polymorphic species of the genus *Colocasia* and 'antiquorum' as a variety (Hill, 1939). Commonly cultivated taro belong to two botanical varieties; *Colocasia esculenta* (L.) Schott var. 'esculenta' and *Colocasia esculenta* (L.) Schott var. 'antiquorum' (Purseglove, 1973).

C. esculenta var. 'esculenta' has edible underground stems called corms and bud-like smaller structures called cormels. Above ground, the leaves and petioles of certain cultivars are also consumed (Alercia, 2013). In comparison, *C. esculenta* var. 'antiquorum' has a small globular central corm, with several relatively large cormels arising from this inner corm. *C. esculenta* var. 'esculenta' is agronomically referred to as the dasheen type of taro while *C. esculenta* var. 'antiquorum' is called the eddoe type. The majority of taro cultivated in the Asia-Pacific region is the preferred dasheen type (Onwueme, 1999).

Due to the widespread distribution of taro, there are many cultivars grown throughout the world. These cultivars may be differentiated based on physiological characteristics such as shape and size of the corm or shape, size, color and texture of the leaves and petioles. Additionally, agronomic or culinary predilection may also be used to distinguish cultivars (Onwueme, 1999). Historical accounts ascertain that Hawaiian people grew approximately 300 cultivars of taro; both wetland and dryland types even before first contact with Europeans in 1778. These cultivars were segregated on the basis of leaf color variation and their ability to grow in specific conditions and locations (Cho, Yamakawa, & Hollyer, 2007; Jacobs, 2011). Whitney et al.

(1939) referring to MacCaughey and Emerson (1914) stated that approximately half of these 300 varieties are duplicates.

1.2 Origin and Distribution

Taro is thought to have originated in South Central Asia, most likely in India or the Malay Peninsula (Hill, 1939; Whitney et al., 1939). Conversely, a karyotypic study points at North-eastern India as the center of origin of taro (Kuruvilla & Singh, 1981). From its source, taro spread eastwards to the rest of South East Asia, China, Japan and the Pacific Islands with the early seafaring voyagers. At the same time, it was taken westward to Egypt and the eastern Mediterranean; and then southward and westward to East and West Africa and likely introduced to the Caribbean and the Americas from there (Lee, 1999; Matthews, 1995, 2004; Onwueme, 1999; Plucknett, 1983).

Interestingly, an isozyme variation study of taro cultivars from Asia and Oceania identified a higher level of genetic diversity in Asian taro while pointing to a distinctive gene pool in the Pacific. Indonesian cultivars had the highest genetic variation and results indicated Pacific cultivars may have originated from a narrow base within this gene-pool (Lebot & Aradhya, 1991). Noyer et al. (2003) observed similar differentiation between Southeast Asian and Melanesian taro using a simple sequence repeat (SSR) marker technique thereby confirming the isozyme results. These two observations were further supported by Kreike, Van Eck, & Lebot (2004) with the use of amplified fragment length polymorphism (AFLP)-PCR. Phylogenetic analysis from their research virtually conclusively separated 255 taro accessions into Asian and Pacific gene pools. These results elucidate possibly two separate domestication

processes and Deo et al. (2009) suggested that taro can therefore be recognized as a native plant of the Pacific as well.

Polynesian migration into the Hawaiian Islands is regarded as the primary source of introduction of *C. esculenta* into Hawaii (Greenwell, 1947). Of the hundreds of taro cultivars developed by Hawaiians prior to European arrival, 84 were documented in 1939 by Whitney et al.; information on which is accessible at this University of Hawaii at Manoa (UHM) - College of Tropical Agriculture and Human Resources (CTAHR) online catalogue: http://www.ctahr.hawaii.edu/site/Taro.aspx. The bulk of the taro produced in Hawaii now is dominated by the varieties 'Maui lehua' which has extensively overtaken the once-preferred 'Lehua Maoli'; then 'Moi' and 'Bun Long'(Cho et al., 2007). The widespread cultivation of only a few selected varieties has been attributed to large scale commercial farming and research work that promote certain varieties over another. The notion exists that taro in Hawaii has been transformed from a commodity once sacrosanct and grown widely throughout the state to merely a crop mono-cultured in non-traditional ways (Jacobs, 2011).

1.3 Morphology and Anatomy

Taro, which has been referred to as the "potato of the tropics," is an herbaceous perennial plant that grows 1 – 2 m tall and covers a similar area with the spread of its canopy. A detailed taxonomic description was documented by Whitney et al. (1939):

> "Extremely variable, succulent, glabrous herb, 4 to 18 dm. tall. Stem a subterranean corm with scaly outer bark and thin, usually highly colored cortex, single or branching from the apex, with conspicuous leaf-scar rings, producing cormels (aha) or rhizomes as offshoots. Petioles 4 to 18 dm. long,

> erect or spreading, sheathing at base with sinus to about midway, uniformly light or dark green to variously highly colored, striped, or flecked. Blades 25 to 85 cm. long, 20 to 60 cm. wide, usually peltate, ovate to more or less sagittate, the apex acuminate, a dark-colored spot known as pikå on the upper surface at the point of junction with petiole. Inflorescences 2 to 5 together in the leaf axils, the peduncles 15 to 50 cm. long, each spadix enclosed within a spathe. Spathes oblong-lanceolate, divided by a transverse constriction into two unequal parts, the lower part 3 to 5 cm. long, loosely or tightly convolute, more or less fleshy, tubular, the upper part 15 to 35 cm. long, usually tightly but sometimes loosely convolute, lanceolate. Spadix 6 to 14 cm. long, with female flowers at the base, consisting of a few obovoid or ellipsoid ovaries 0.5 to 1.5 mm. in diameter, the stigma sessile, capitate; constricted above the female flowers and beset over a length of 2 to 5 cm. with light yellow sterile flowers; above the sterile flowers and over a length of 2 to 4 cm. beset with male f1owers,consisting of 2 to 6 sessile anthers which are fused into an obconical synandrium; with yellow constricted, obtuse or acute sterile appendage at apex. Fruit a berry, 3 to 5 mm. across, ellipsoid. Seed 1.2 to 1.5 mm. long, 0.7 to 1 mm. wide, hard, ovoid."

The underground consumable central corm represents the main stem structure of the plant. In the dasheen type of taro, which is the subject of this study, the corm is cylindrical and large; shaped like a top with rough ridges, lumps and fibrous gangling roots. The corms can grow up to 30 cm long and 15 cm in diameter, generally weighing around 1-2 pounds, but can weigh up to eight pounds (Moore & Lawrence, 2003; Onwueme, 1999).

The taro corm and almost all other parts of the plant contain idioblasts (cells with raphides or bundles of calcium oxalate crystals). These compounds are responsible for the acridity or itchiness of taro and for this reason it is not recommended to consume any part of the plant uncooked. The density and woodiness of the corm increases with age. The skin covering

the corm is brown with white or pink flesh. Certain varieties of *C. esculenta* are known to produce smaller tubers or cormels which grow off the sides of the main corm. The cormels are also edible. Although rare, some taro plants shoot off runners as well. These grow horizontally along the soil surface for a short distance, rooting down at intervals to give rise to new standing plants (Moore & Lawrence, 2003; Onwueme, 1999).

2.0 Ethnobotany of Taro

2.1 Food Security, Nutritional and Economic Importance of Taro

Taro is one of the major staple root-crops in all Pacific island countries (PICs), as such integral to the region's food security, nutrition and economy. Historically, taro topped the ranks as the most important crop grown throughout the Hawaiian Islands (Moore & Lawrence, 2003). Although it no longer holds that status in comparison with crops such as nuts, seed corn and coffee in present-day Hawaii (Gomes, 2012), taro is still consumed in considerably large quantities throughout the PICs and in a variety of ways.

Taro corm, the main edible part of the plant is boiled, baked or fried and consumed with an array of vegetable and meat preparations. In what is a favorite Pacific cuisine, taro along with other staple crops and meat is cooked on hot stones in dug-out earth ovens, known as *imu* in Hawaii, *umu* in Samoa and *lovo* in Fiji (Deo et al., 2009). This is a common preparation method employed during traditional ceremonies, festivities and celebrations. Young taro leaves cooked with coconut cream, canned meat or tuna, called *palusami* is another delicacy – a rich side dish prepared in the *imu, umu* or *lovo*. Fiji-Indians make curry from both the corm and the leaves of the taro plant. The inflorescence is also a delicacy in some Asian and Pacific food cultures (Rao et al. 2010).

In Hawaii, taro was "the staff of life" for early settlers (Wang & Higa, 1983; Whitney et al., 1939) made into the traditional dish *poi* (Potgieter, 1940) – a fermented or unfermented paste achieved by mashing cooked corm with water to a desired consistency (Lee, 1999). Another authentic Hawaiian dish, *lu'au,* has been passed down from generations; made by cooking young tops and leaves with coconut cream and *poi*, the sour paste made from boiled taro corms (Uchida et al., 2008). Commercially, taro has long been processed into flour and beverages (Payne, Ley, & Akau, 1941) and more recently into taro chips (Hollyer, Paull, & Huang, 2000) in several PICs. Taro flour, to some extent, is also utilized in the pastry and baking industry (Ammar, Hegazy, & Bedeir, 2009). Even then, the intensity of taro cultivation has dropped over the years and taro is now regarded as a highly nutritious yet under-utilized commodity (Alercia, 2013); nonetheless certainly one that is vital for the region's food security.

Nutritionally, the taro corm is rich in complex carbohydrates and is a primary source of starch, the dietary contribution of which is similar to potatoes. Taro starch has a digestibility value of 98.8 percent owing to the minute size of its granules which are ten times smaller than that of potato starch (Jane et al., 1992). For this reason, taro flour is an ideal constituent for the production of infant formulae and is also an important ingredient of canned baby foods. Additionally, as a substitute, taro is beneficial for persons with digestive problems and allergies to cereals; and for children sensitive to milk (Lee, 1999; Moore & Lawrence, 2003). Taro leaves are rich in protein while the corm has very little fat and contains dietary fiber. The leaves and corm contain several essential vitamins such as E, C (ascorbic acid), B1 (thiamin), B2 (riboflavin), B6, B9 (folic acid) and macro and micro minerals; namely magnesium, phosphorous, copper, potassium, calcium, iron and manganese (Alcantara, Hurtada, & Dizon, 2013; Alercia, 2013; Payne et al., 1941; Potgieter, 1940; Temesgen & Retta, 2015).

Alternatively, researchers have also recommended the use of taro corms, leaves and petioles as important ingredients in animal feed (Adejumo, Babalola, & Alabi, 2013; Moore & Lawrence, 2003).

Furthermore, the taro plant has a variety of traditional and herbal medicinal purposes. In Africa, fresh leaves are used for blood clotting, soothing wounds, sores and boils while in Indonesia juice from the stalk is employed to neutralize snake poison and root extract is used to treat rheumatism and acne (Abbas, 2011). In the Philippines, leaves and corms were prepared in special ways to help ease pain during childbirth. Some tribes used early morning dew on the leaves as eyewash, sitting on taro leaves helped women with dysmenorrhea while the styptic petiole juice was used to arrest arterial hemorrhage. Taro plants also provided remedy for ear aches and were used as laxative to treat hemorrhoids or as antidotes for stings. Mixing tuber ash with honey helped cure mouth apthae. In Hawaii, people suffering from fever were made to drink raw taro leaf juice mixed with sugar for relief. Taro provided remedies for many other illnesses including constipation and tuberculosis (Moore & Lawrence, 2003). *Poi* has been shown to benefit infants with food allergies and treat "failure-to-thrive" conditions. It also has use as a probiotic (Brown & Valiere, 2004).

Taro is one of the few unique commodities of which both the above and below-ground parts are consumed. It is therefore an important domestic market fresh crop in almost all PICs (McGregor et al., 2011). Revamped production in recent years has also enabled a few countries in the region, namely Fiji, Samoa, Tonga and Vanuatu to open export markets for their taro (Sami, 2011). FAO assessments show that between 2005 and 2009, Pacific countries produced on average a combined 430,000 tonnes of taro. During that same period, 10,000 to 13,000 tonnes of taro were exported annually to countries such as Australia, New Zealand, USA and Japan.

Fiji, the highest exporter accounted for almost 95 percent of total exports valued around FJD20 million per annum (McGregor et al., 2011). The export figures seem low compared to the total production figures because Papua New Guinea (PNG), the largest grower of taro in the Pacific (representing over half of the total production) favors domestic sale. Additionally, shipping costs to trading partners are high meaning it cannot offer taro at competitive prices like Fiji (McGregor et al., 2011). Locally, Hawaii produced 3.1 million pounds (1,406 tonnes) of taro in 2013 worth over USD1.9 million ("USDA Crop Production 2013 Summary," 2014, "USDA Crop Values 2013 Summary," 2014).

2.2 Socio-cultural Significance of Taro in Hawaii and the Pacific Islands

Taro has its roots entrenched deep into Pacific history and has impacted daily life like no other crop has, particularly in the Polynesian countries. For native Hawaiians, daily routines revolved around the *lo'i* (flooded taro patches) and taro farming formed the basis of early civilization (Greenwell, 1947). Over time taro has attained significant socio-cultural importance not only in Hawaii but other PICs as well, some of which are discussed below.

Taro cultivation was not merely an activity for ensuring food availability in early Hawaii; it had strong attachment to beliefs about creation. This is portrayed in the Hawaiian creation chant – *Kumulipo.* The chant is a narrative of how taro is the plant from which Hawaiians have descended. The *Kumulipo* conveys the legend of *Papa,* Earth Mother and *Wakea,* Sky Father who gave birth to Ho'ohokukalani, the most charming and endearing woman of all times. Ho'ohokukalani's first child, Haloa-naka, was still born hence entombed in the earth. From the site where Haloa-naka was buried germinated the *Kalo* or taro plant. This plant provided sustenance to the second born; Haloa-naka's younger brother, Haloa. Because taro is

vegetatively propagated, Haloa-naka lives on. In this way, the taro plant is superior and more sacred than man himself; it is an elder sibling deserving care and respect. Hawaiians delineate their ancestry from the younger son Haloa (Cho et al., 2007; Jacobs, 2011; Uchida et al., 2008).

In early Hawaii, prayers were offered to the Gods to seek blessings every time a new activity commenced during the farming cycle. These occurred right from land preparation to harvesting the *kalo* and consumption. The venerations dignified the act of farming and provided significance to the work done to produce taro. Intertwined with social-norms, the agricultural system of the past was not designed for profit making. It rather focused on feeding communities, maintaining religion, practicing tradition and upholding customary obligations (Uchida et al., 2008). This symbolism and spiritual connection conferred an ambience of mythology upon the taro plant and its history in Hawaii ("Taro," 2014). Although the industry has been commercialized, this representation continues to exist. Art in various forms centered around the theme of *kalo* are visible throughout the state, a testimony to how taro continues to be an influence in Hawaii's food and agricultural landscape (Deo et al., 2009).

An enthralling evidence to this emotional attachment to taro is the case of patenting efforts by UHM – CTAHR's plant pathologist Eduardo Trujillo who introduced three improved taro varieties in 2002 after an extensive breeding program started in the early 1990s ("CTAHR and Taro," 2009). These varieties were resistant to taro leaf blight (TLB) and also possessed desired traits from the local cultivar Maui Lehua (Trujillo, Menezes, Cavaletto, Shimabuku, &Fukuda, 2002). Maui Lehua is one of the cultivars in the royal Lehua family of Hawaiian cultivars. The displeasure of some factions of the local community with regard to the indigenous and cultural sentiments attached to taro versus ownership of the new varieties by the university resulted in protests in 2006. Consequently, UHM renounced its patent rights and made the

varieties available publicly ("CTAHR and Taro," 2009). *Kalo* was designated the state plant of Hawaii in 2007. Following this announcement, the Hawaii County Council issued a ban on genetically modified taro (and coffee) on Big Island in 2008 by passing a bill which made it illegal to "test, propagate, cultivate, raise, plant, grow, introduce or release genetically engineered taro and coffee" in the county. Citing taro as a sacred plant, the Maui County Council followed a similar path in 2009 (Jacobs, 2011).

Elsewhere in the Pacific, Palauans also have a strong affiliation with the taro plant and believe in an age-old proverb that states "the taro patch is the mother of our breath". Taro is not only a source of food and income in Palau, but a token of exchange and gratitude during cultural customs and family events such as birth ceremonies and funerals (Bishop, 2013). Similarly, taro is superior over all other root crops and the preferred commodity for royalty, gift-giving, traditional feasting and the fulfillment of social obligations in Fiji (Vilsoni, 1993), Samoa (Taotua, 1993), Tonga (Pole, 1993), the Solomon Islands (Liloqula, Saelea, & Levela, 1993), Micronesia (Primo, 1993) and many other countries in the South Pacific (Sivan & Liyanage, 1993). Cultivated in the region for centuries, taro is also mentioned in the oral folklore, myths and legends of many Pacific cultures (Deo et al., 2009). Taro inherently is a symbol of cultural identification synonymous to Pacific lifestyle and the people, wherever they live in the world (Onwueme, 1999). The socio-cultural prominence of taro across the PICs is unmatched by any other root crop or agricultural commodity and this prestige will continue to persist having endured the test of time after first being domesticated in the region thousands of years ago.

3.0 Known Pests and Diseases of Taro

3.1 Pests

Taro beetles, *Papuana* spp. (Coleoptera: family Scarabaeidae) are perhaps the most important pests of taro in the South Pacific region. These shiny black scarab beetles, about 15-25 mm in size at the adult stage have been reported to cause extensive damage to the corm in PNG, Solomon Islands, Vanuatu, Kiribati and Fiji. Other aroids in the taro family are also vulnerable to taro beetle attack (Aloalii et al. 1993). Burrowing action into the corms creates tunnels and exposes the flesh, providing entry points for other pathogens. This secondary rotting is a major contributor to corm spoilage in the field as well as in post-harvest storage (Lal et al., 2008). Taro beetles have not been reported in Hawaii, however, globalized trading routes and human movement do present the risk of accidental introduction. Quarantine efforts are therefore paramount in keeping this pest from arriving into the state.

Conversely, a pest of concern in Hawaii is the taro root aphid, *Patchiella reaumuri*. It was first discovered on Big Island (Hawaii) in 1971 then on Oahu in 1995. The taro root aphid is particularly destructive for dryland production but has not been problematic in wetland taro. It is thought to be host specific, presumably feeding only on taro and a few related plants within the family. While the aphids are yellow-gray in color, their colonies are usually covered with a mass of fine, white, cottony, waxy substance. Winged sexual types of the taro root aphid have not been detected in Hawaii; reproduction occurs without male fertilization. Heavy losses of 75-100 percent in certain varieties have been reported as a result of taro root aphid infestation. Damage is escalated by drought conditions and is severe on young plants (Sato et al. 1997).

Other pests of concern include the taro planthoppers (*Tarophagus* spp.), heavy infestation by which may result in the wilting and death of plants; hawk-moth (*Hippotion celerio*), the

larvae of which may heavily feed on the leaves causing defoliation; and the armyworm caterpillars (*Spodoptera litura*) that also consume the leaves (Carmichael et al., 2008; Deo et al., 2009). Spiraling whitefly, *Aleurodicus dispersus*, the aphid, *Aphis gossypii* and mealybugs (Family Pseudococcidae) are also common pests. Widely distributed across the Pacific, their piercing and sucking action can also cause wilting and death in heavy infestations. Exudated honeydew promotes the growth of sooty moulds that impede photosynthesis. Tobacco whitefly, *Bemesia tabaci*, has also been noted for similar, but less common damage. Additionally, spider mites (*Tetranychus* spp.) cause whitish-yellow speckling and premature death of leaves (Carmichael et al., 2008) while mealybugs have long been linked to *Taro bacilliform virus* (TaBV) transmission (Gollifer et al., 1977; Macanawai et al., 2005).

Finally, snails round up the list of pests for taro. The giant African snail (GAS), *Lissachatina fulica*, found in many PICs has been observed feeding on taro leaves. Carmichael et al. (2008) report that the level of damage is directly related to the population; in areas where numbers are low, GAS feed on alternative, more preferred plants such as garden cabbage. Apple snails, *Pomacea canaliculata* present on all islands except Molokai and Lanai and *Pila conica* present only on Molokai are major threats to Hawaiian taro. They were introduced from South America for the Hawaiian aquaculture industry, however, escaped and began appearing in taro patches in 1983 – 1984. These snails are rapid invaders and infest in large numbers, hence damage significant taro foliage resulting in reduced leaf and corm yield (Levin, 2006; Martin, 2004). The snails also pose risk of injury and are annoying to workers in *lo'i*.

3.2 Nematodes

The nematode *Hirschmanniella miticausa* causes the unique "miti miti" disease and was first reported out of the Solomon Islands in 1983 (Bridge, Mortimer, & Jackson, 1983). This species of nematode is also found in PNG. *H. miticausa* infestation results in dry brown rot of the corm, about 1 – 10 mm in size that originate from the base of the corm. It is nearly impossible to identify the rot symptoms until corms are harvested – chlorosis and wilting of leaves are indicators (Carmichael et al., 2008). Root knot nematodes (*Meloidogyne* spp.) and lesion nematodes (*Pratylenchus coffeae*); fairly widely distributed across the Pacific cause roots knots and root decay, stunting and death in taro respectively. Substantial corm yield losses have been reported (Arakaki, 1993; Sipes & Arakaki, 1997; Torigoe, Fukunaga, & Muta, 2002).

3.3 Fungal Diseases

There are numerous diseases produced by fungi important in taro production. One of the most common throughout the PICs is corm rot caused by the soil-borne fungus *Athelia rilfsii*. It is also the pathogen for a post-harvest pinkish rot on the corms. Brown leaf spot, also known as cladosporium leaf spot caused by *Cladosporium colocasiae* and Orange leaf spot caused by *Neojohnstonia colocasiae* are also widespread throughout the Pacific. Brown leaf spot is sometimes referred to as ghost spot due to the characteristic reddish-brown spots or blotches being less pronounced on one side of the leaf surface (Carmichael et al., 2008; Parris, 1941).

Spongy black rot, caused by *Lasiodiplodia theobromae* (previously *Botryodiplodia theobromae*), has been reported to infect taro in Guam, PNG, Samoa and the Solomon Islands while white spot of taro, caused by *Leptosphaerulina trifolii* is found in American Samoa, PNG, Samoa, Solomon Islands and Tuvalu. Both fungi have been detected on other hosts in majority of

the countries in the region. Corm and leaf spot as a result of *Marasmiellus stenophyllus* infection has only been recorded in American Samoa, Tahiti, Wallis and Futuna and on a different host in Fiji. The "shot hole" fungi, *Phoma* spp. occurs throughout the Pacific while the leaf blotch fungus *Pseudocercospora colocasiae* is more restricted in distribution and has been observed only in American Samoa, Fiji, New Caledonia, Samoa, Solomon Islands, Tahiti and Vanuatu (Carmichael et al., 2008). Locally, phyllosticta leaf spot (*Phyl1osticta colocasiophila*) has long been recognized to affect dryland taro. *Ceratocystis fimbriata* (black rot), *Rhizopus stolonifera* (rhizopus rot) and *Fusarium solani* or other *Fusarium* spp. (fusarium dry rot) are taro pathogens as well (Ooka, 1990). Southern blight (*Sclerotium rolfsii*) has also been detected in Hawaiian taro (Parris, 1941).

3.4 Oomycete Diseases

The most important disease produced by these fungus-like eukaryotic microorganisms is taro leaf blight (TLB), caused by *Phytophthora colocasiae* which was first reported out of Java, Indonesia by Marian Raciborski in 1900. It is a highly destructive pathogen that primarily attacks the leaves; petioles and corms are also prone to infection (Brooks, 2008). Corm yield losses of 25-50 percent in the Pacific and 25-35 percent in the Philippines have been reported. Susceptible varieties in Hawaii have been noted to lose 95 percent of leaf yield once infected (Brooks, 2005; Nelson, Brooks, & Teves, 2011). TLB decimated Samoan and the American Samoan taro industries during an outbreak in 1993-1994. Within two years, Samoa's annual USD10 million industry plummeted to just a little over USD60,000 (0.5% of average annual export value). American Samoa, which was producing close to 400,000 kg of taro before the epidemic managed only 5,000 kg in 1995 (Brooks, 2008; Singh et al., 2012). TLB is believed to have been first

reported in Hawaii in 1920, two years after being discovered in Guam in 1918 (Hunter, Pouono, & Semisi, 1998). It is now spread throughout the Pacific and also found in Asia, East Asia, Africa, the Caribbean and the Americas (Nelson et al., 2011).

Another important oomycete disease is pythium rot, also known as corm soft rot caused by many species within the genus *Pythium*. This disease was first reported in Hawaii in 1902 by Sedgwick. It may start at the base or side of the corms then proliferate upwards infecting the entire tuber. Parris (1941) reported that the color of the rots vary in diseased corms; from whitish yellow to shades of grey or blue to dark purple. This rotting affects the aboveground appearance of the plant making it look stunted as it slowly dies away (Carmichael et al., 2008). *Pythium aphanidermatum, P. graminicola* and *P. splendens* have been reported in Palau, Samoa and Hawaii associated with yield losses of up to 80 percent. *P. myriotylum* has also been found in diseased corms in Solomon Islands, Samoa and Hawaii. *P. irregulare* has only been reported out of New Caledonia and *P. carolinianum* only out of Hawaii (Ooka, 1990).

3.5 Bacterial Diseases

Bacterial soft rot caused by *Erwinia chrysanthemi* has been recorded in the Solomon Islands (Carmichael et al., 2008). When infected, taro corms develop an aqueous soft decay whitish to dark blue in color. The rot is accompanied by a pungent odor. Bacterial leaf spot, the result of *Xanthomonas axonopodis* (previously *X. campestris*) pv *dieffenbachiae* infection is also present in Hawaii, however, the disease is not significant (Ooka, 1990).

3.6 Viral Diseases

Several viruses are important pathogens of *Colocasia* taro. One of the most destructive viral diseases of taro in the Pacific is 'alomae'. Gollifer & Brown (1972) were the first to describe this disease which is confined to the Solomon Islands and PNG. Plants affected by alomae develop a feathery mosaic symptom, emerging leaves are crinkled and fail to open properly; the plant overall is stunted with thickened, twisted dark green leaves. It is thought that co-infection by *Colocasia bobone disease virus* (CBDV) and *Taro bacilliform virus* (TaBV) causes this disease (James, Kenten, & Woods, 1973; Ooka, 1990) while *Taro vein chlorosis virus* (TaVCV) has also been suggested as a possible factor in the etiology of this disease (Carmichael et al., 2008). Substantial yield losses occur as a result of plant death (Gollifer et al., 1978). Of the two viruses initially associated with alomae, CBDV is transmitted by the delphacid *Tarophagus proserpina* and TaBV by the mealybugs *Planococcus citri* and *Pseudococcus longispinus* (Gollifer et al., 1977; Macanawai et al., 2005).

Similarly destructive on its own is CBDV, a putative rhabdovirus found only in PNG and the Solomon Islands. It is relatively widespread and causes the bobone disease (when not involved with TaBV – in that case alomae results). Plants suffering from bobone display severe stunting, distorted, thickened and stiff leaves. Galls may also develop on the petioles and larger leaf veins. Some researchers believe alomae and bobone can simultaneously be seen on taro plants and this condition is attributable to the Alomae-Babone virus complex (ABVC) (Ivancic et al., 1993). Recently, Higgins et al. (2016a, 2016b) reported the full genome sequence of a Colocasia bobone disease-associated virus (CBDaV) from taro affected by the babone disease in Solomon Islands. Phylogenetic analysis places this 12,193 nt long negative-strand RNA virus in the *Cytorhabdovirus* genus and it is believed this sequence is that of CBDV. A less destructive

yet most widespread virus of the taro family in the PICs is the *Dasheen mosaic virus* (DsMV). First described in 1970 by Zettler et al., infected plants display an array of mosaic patterns on the leaves with mild to moderate distortions. DsMV, member of the genus *Potyvirus* is a stylet-borne (non-persistent) virus transmitted by several species of aphids, namely, *Myzus persicae*, *Aphis craccivora* and *A. gossypii* (Ooka, 1990).

TaBV, genus *Badnavirus,* also known as 'Taro badnavirus' is common in many countries throughout the Pacific region. The virus was first described by James et al.(1973) and the full viral genome was sequenced three decades later (Yanget al. 2003). TaBV produces latent, erratic vein yellowing, often near the leaf margin. Leaf blades may noticeably bend backwards and sometimes creasing is apparent (Carmichael et al., 2008). Just last year, deep sequencing of small RNAs (sRNAs) from taro plants with *Badnavirus* infection-like symptoms from China led to the discovery of a new virus in this genus affecting taro – tentatively called the Taro bacilliform CH virus (TaBCHV) (Kazmi et al., 2015).

TaVCV is another understudied taro viral disease of the Pacific. First described in 1999 by Pearson et al., its genome was fully sequenced in 2005 by Revill et al. and TaVCV gained formal recognition as a member of the genus *Nucleorhabdovirus* in the family *Rhabdoviridae.* It has so far been reported out of 9 countries in the Central/South Pacific region; namely the Federated States of Micronesia, Fiji, New Caledonia, Palau, PNG, Solomon Islands, Tuvalu, Vanuatu and most recently in the United States of America in the state of Hawaii. Taro leaves infected by TaVCV show a characteristic feather-like chlorosis. This feathery yellowing broadens between adjacent veins as the leaf ages. The chlorosis coalesces into a network and in later stages may become necrotic leading to a "tattered appearance" of the leaf margins. These symptoms usually show up at maximum growth, hardly ever on young plants. The vein-chlorosis

produced by TaVCV is more expressive than TaBV induced chlorosis. Petiole streaking occurs in some varieties while stunting has also been observed, but rare. Taro plants do not experience gall formation as a result of TaVCV infection which is typical of CBDV, another rhabdovirus (Carmichael et al., 2008). A fifth taro virus, Taro reovirus (TaRV) has also been reported out of PNG, Solomon Islands and Vanuatu, however, it's etiology and symptomatology are not well understood (Revill et al., 2005b). A few years back, *Groundnut bud necrosis virus*, GBNV (genus *Tospovirus,* family *Bunyaviridae)* was also found infecting taro in India. GBNV causes mosaic, chlorotic spotting and necrotic flecking on the leaves; the infected plant is overall stunted (Sivaprasad et al., 2011). This, however, has been an isolated case and new reports of GBNV infection on taro have not been published elsewhere.

4.0 *Taro vein chlorosis virus* (TaVCV) (genus *Nucleorhabdovirus*, family *Rhabdoviridae*, order *Mononegavirales*)

4.1 *Rhabdoviridae* family of viruses

Rhabdoviridae is a virus family within the order Mononegavirales which also contains the *Bornaviridae*, *Filoviridae, Mymonaviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae* and *Sunviridae* families. There are 13 assigned genera within the *Rhabdoviridae* family. These include C*ytorhabdovirus* (10 species), *Dichorhavirus* (2 species), *Ephemerovirus* (5 species), *Lyssavirus* (14 species), *Norvirhabdovirus* (4 species), *Nucleorhabdovirus* (10 species), *Perhabdovirus* (3 species), *Sigmavirus* (7 species), *Sprivivirus* (2 species), *Tibrovirus* (2 species), *Tupavirus* (2 species), *Varicosavirus* (1 species) and *Vesiculovirus* (9 species). This family of viruses also includes 4 species who have not been assigned a genus ("International Committee on Taxonomy of Viruses," 2015).

Table 1.1. Members of the plant infecting genera *Cytorhabdovirus, Dichorhavirus* and *Nucleorhabdovirus* within the *Rhabdoviridae* family.

Family	Genus	Species
Rhabdoviridae	*Cytorhabdovirus*	*Alfalfa dwarf cytorhabdovirus*
		Barley yellow striate mosaic virus
		Broccoli necrotic yellows virus
		**Colocasia bobone disease associated virus
		Festuca leaf streak virus
		**Lettuce necrotic yellows virus*
		Lettuce yellow mottle virus
		Northern cereal mosaic virus
		Sonchus virus
		Strawberry crinkle virus
		Wheat American striate mosaic virus
	Dichorhavirus	*Coffee ringspot virus*
		**Orchid fleck virus*
	Nucleorhabdovirus	*Datura yellow vein virus*
		Eggplant mottled dwarf virus
		Maize fine streak virus
		Maize Iranian mosaic virus
		Maize mosaic virus
		**Potato yellow dwarf virus*
		Rice yellow stunt virus
		Sonchus yellow net virus
		Sowthistle yellow vein virus
		Taro vein chlorosis virus

*Indicates type member of its genus. Adopted from: ICTV, online
**Indicates a tentative member not formally recognized by ICTV

Species of only three of the 13 genera; *Cytorhabdovirus*, *Dichorhavirus* and *Nucleorhabdovirus* infect plants (Walker et al., 2000) (Table 1.1). *Cytorhabdovirus* and *Nucleorhabdovirus* are differentiated based on the sites of replication and maturation of virus particles with the plant cell. Nucleorhabdoviruses multiply in the nucleus of host cell then accumulate in the perinuclear space, whereas Cytorhabdoviruses characteristically replicate and accumulate in the cytoplasm (Jackson, Francki, & Zuidema, 1987). Species within each of these two genera are segregated depending on the hosts they infect and vector specificity. Molecular and serological assays and now more common genomic sequence data are used as additional tools for species demarcation (Jackson et al., 2005). The main distinguishing feature of the *Rhabdoviridae* family is the enveloped virions with bacilliform or bullet-shaped morphology. The single stranded negative sense RNA are encapsulated in these particles, size estimates of which range from 45 to 100 nm in width and 130 to 350 nm in length (Brown, 1987). However, the genus *Dichorhavirus* is an exception. Members of this genus have un-enveloped particles and bi-partite (segmented) genomes (Kondo et al., 2006). They are vectored by the *Brevipalpus* mites (family Tenuipalpidae). The *Orchid fleck virus* (OFV) is the type member of this genus (Dietzgen et al., 2014).

All rhabdovirus genomes seemingly code for five major viral proteins flanked by the leader (l) and trailer (t) regions in the order 3'-l- N, P, M, G, L -t-5' in negative polarity. The first open reading frame (ORF) upstream of the 5' end encodes for the large L protein; conserved domain for the RNA dependent RNA polymerase (RdRp) which is responsible for the replication of the viral RNA and transcription. Upstream of this, the G ORF encodes for the glycoprotein. This is a structural protein which forms the spike-like projections on the surface that aids virus attachment to the infected cells. From the 3' end, the N ORF encodes for the nucleoprotein;

major constituent of the viral nucleocapsid. Downstream to this is the P ORF coding for the phosphoprotein (also called the M1 protein) which is a component of the viral polymerase. Further downstream is the M ORF which codes for the matrix (or M2) protein. These form the inner lining of the virus envelope and play a role in virus budding. Plant rhabdoviruses that have been fully sequenced so far contain one to four additional ORFs. These extra genes mostly occur at position X between the P and M genes or alternatively at position Y between the G and L genes (Healy, Banyard, & Fooks, 2013; "International Committee on Taxonomy of Viruses," 2015; Jackson et al., 1987; Redinbaugh & Hogenhout, 2005; Walker et al., 2015, 2000).

4.2 The genus *Nucleorhabdovirus*

Nucleorhabdovirus is one of the three plant virus genera in the family *Rhabdoviridae*. This genus was previously classified as Plant Rhabdovirus group B. Species in this genus have bacilliform or bullet shaped enveloped, usually straight particles inside which the unsegmented ssRNA genome is packaged (Fig. 1.1). Upon infection, the virions replicate and mature in the nucleus of the host plant cell. The *Nucleorhabdovirus* genome is characterized by monopartite, single-stranded, linear, negative sense RNA; 11 kb to 15 kb long. (Brunt et al., 1996).

Virions of this genus are bullet shaped, 170-380 nm long and 55-100 nm in diameter. Prominent spikes or surface projections are strewed evenly over the exterior of the virion. When uncoiled, the nucleocapsids are filamentous, with obvious regular cross-banding and a distinct central canal. The virions are also symmetrically helical with a pitch of 4.2 – 4.7 nm. Typical virion composition is 1 percent nucleic acid, 70 percent protein and 25 percent lipid (Adams & Antoniw, n.d.).

Some of the fully sequenced members of the genus include *Sonchus yellow net virus* (SYNV) (Choi, Scholthof, & Jackson, unpublished data), *Rice yellow stunt virus* (RYSV) (Huang et al., 2003), *Maize mosaic virus* (MMV) (Reed et al., 2005), *Maize Fine Streak Virus* (MFSV) (Tsai et al., 2005), *Taro vein chlorosis virus* (TaVCV) (Revill et al., 2005a), *Potato yellow dwarf virus* (PYDV) (Bandyopadhyay et al., 2010), *Rice transitory yellowing virus* (RTYV) suggested to be synonymous with RYSV (Hiraguri et al., 2010), *Datura yellow vein virus* (DYVV) (Dietzgen, Innes, & Bejerman, 2015) and *Eggplant mottled dwarf virus* (EMDV) (Babaie et al., 2015). All have characteristic six or seven ORFs.

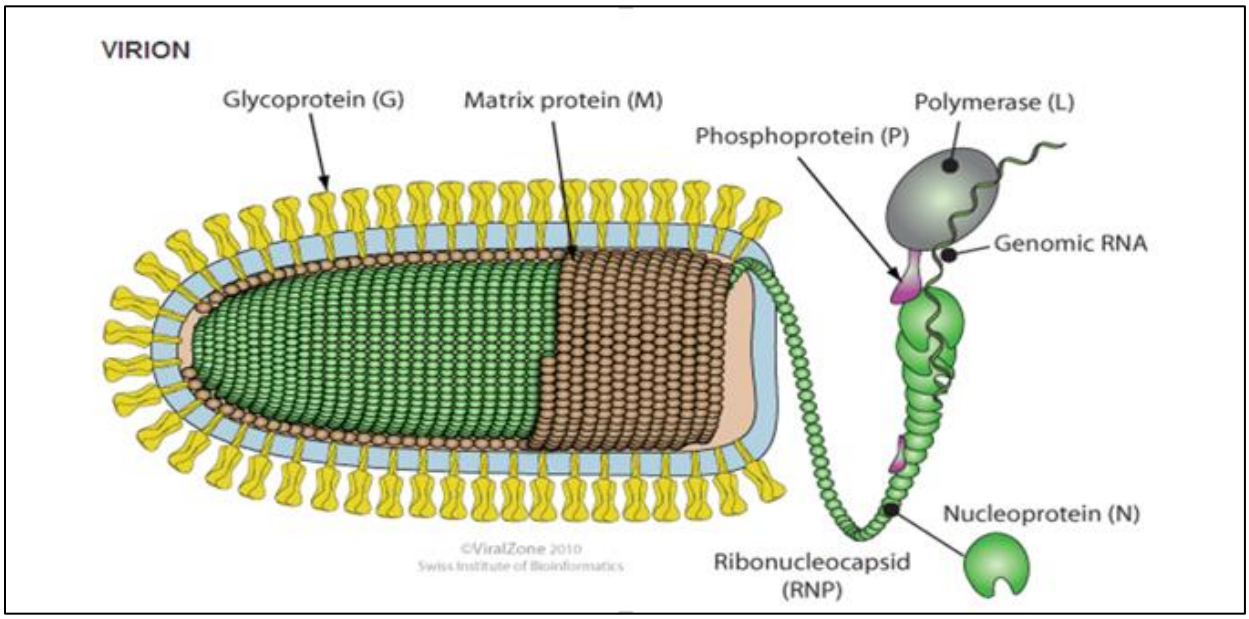


Fig. 1.1. Putative structure of the members of the *Nucleorhabdovirus* genus.

Adopted from: http://viralzone.expasy.org/all_by_species/78.html

4.3 TaVCV – known distribution and history

TaVCV is confirmed to occur in the Federated States of Micronesia, Fiji, New Caledonia, Palau, Papua New Guinea, Solomon Islands, Tuvalu and Vanuatu (Carmichael et al., 2008). The

disease was concurrently first described from Fiji, PNG, Tuvalu and Vanuatu and the name "*Taro vein chlorosis virus*" first coined by Pearson et al. (1999) following comparative analysis against CBDaV, another member of the rhabdovirus family. The distinction was based on recorded differences in particle size, disease symptomatology and serological reactivity.

A 2002 virus survey revealed that TaVCV was relatively widespread in Fiji (Harding, Williams, & Jackson, 2002). The findings of this survey were preliminary and based on symptoms alone; the major objective was to collect fresh leaf tissues from plants exhibiting TaVCV symptoms for virus purification and indexing. Revill et al. (2005a) were the first to characterize the TaVCV genome of a Fijian isolate and confirm the virus as a definitive member of the genus *Nucleorhabdovirus*. Their confirmation was based on the presence of six ORFs in the TaVCV anti-genome; equivalent to the N, P, 3, M, G and L genes common in the *Rhabdoviridae* family. This full genome sequence analysis was supplemented by thin-section electron microscopy data.

In addition to characterizing TaVCV, the group conducted a variability study and claimed high sequence diversity among Pacific strains of TaVCV. Isolates from Fiji, the Federated States of Micronesia, New Caledonia, Papua New Guinea, Solomon Islands and Vanuatu were used for comparison. Furthermore, their analysis showed that TaVCV is most closely related to *Maize mosaic virus* (MMV) – the reference strain for the *Nucleorhabdovirus* genus. The deduced amino acid sequences of the TaVCV ORFs had 63.1 (N), 46.1 (P), 43.4 (gene 3), 46.4 (M), 49.9 (G) and 67.9 (L) percent identity with the deduced amino acid sequences of the respective MMV ORFs. Finally, phylogenetic analysis utilizing the L gene deduced amino acid sequences grouped TaVCV in a clade with all other sequenced *Nucleorhabdoviruses*, separate from the C*ytorhabdovirus* clade.

4.4 TaVCV – symptomatology

An undated online fact sheet authored by Rob Harding; adopted into a 'taro pest guide' produced by the Australian Centre for International Agricultural Research in 2008 provides insights into the symptomatology and effects of the disease exerted on the plant by TaVCV. Infected leaves develop feather-like, striping chlorosis of the veins that often start near the leaf margin. The chlorosis may sometimes display a 'net-like' pattern as well. This distinct vein chlorosis is more expressive than the vein chlorosis elicited by TaBV. The yellowing spreads between the veins and forms a network as the leaf ages. The chlorotic areas in certain cases become necrotic and the leaf margins consequently develop a ragged appearance. Downward bending of the infected leaves is occasionally observed. Some taro cultivars show petiole streaking and overall plant stunting (Fig. 1.2a – g). There is no data reporting the number of leaves that may actually show symptoms once a plant contracts the virus. Field observations indicate that new leaves which emerge after this infected series of leaves appear healthy. Examination of leaves for typical symptoms can provide a basis for preliminary detection, however, symptoms may differ pertaining to cultivar and environmental conditions hence laboratory testing is required.

In comparison to symptoms produced by CBDaV, which is also a rhabdovirus, TaVCV does not produce galls on the leaf blades and petioles of host taro plants. Another unique feature of TaVCV in contrast to other taro virus diseases is that symptoms commonly occur when plants are at maximum growth and not at early stages of the life cycle (Carmichael et al., 2008; Harding, n.d.). TaVCV's recent discovery coupled with the lack of research on this important virus has left many uninvestigated parameters and this is one of the reasons why the effect of this disease on corm yield is unknown.

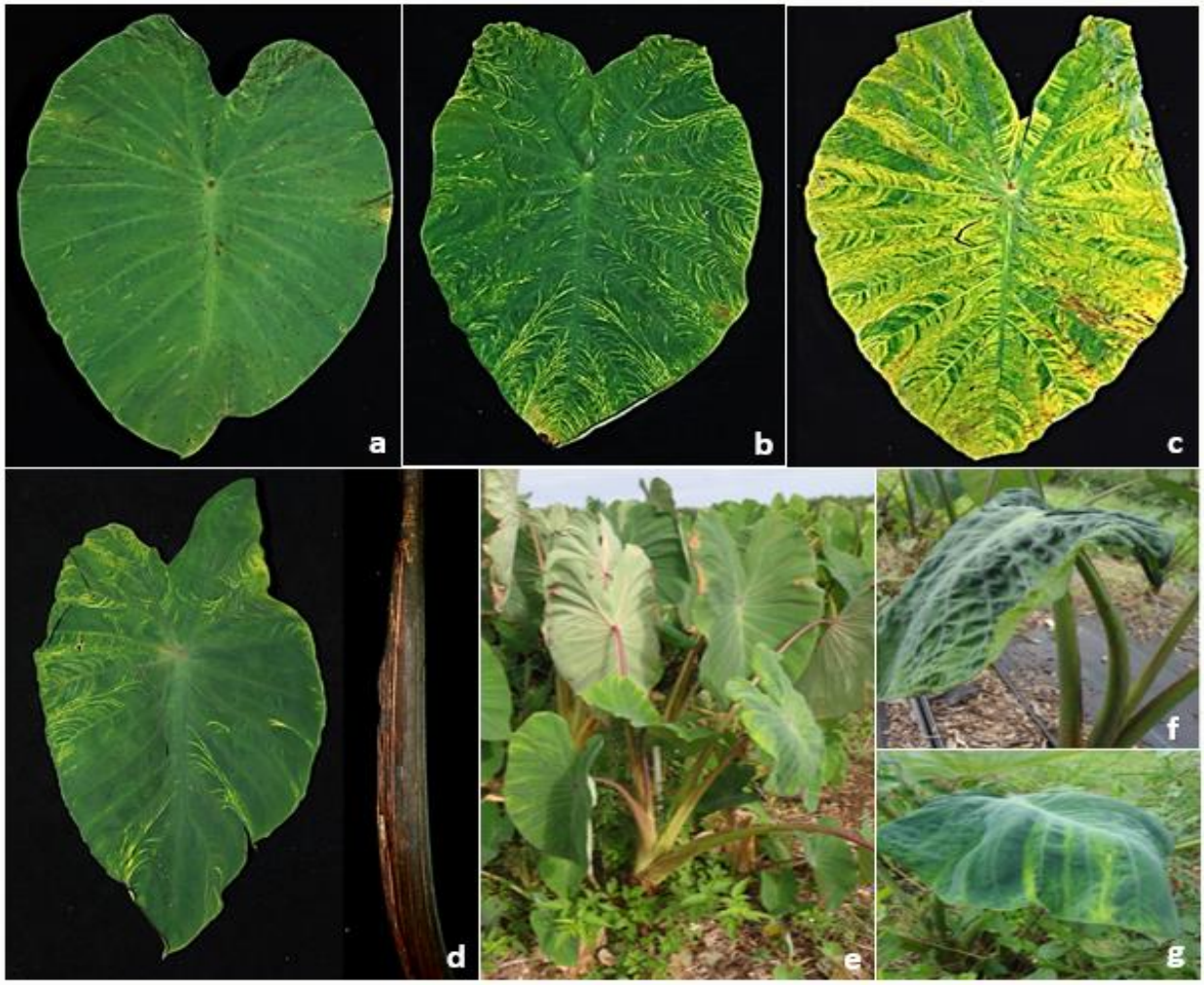


Fig. 1.2a – g. mild (a), moderate (b) and severe (c) feathery interveinal chlorosis of taro leaves. Some cultivars display petiole streaking (d) while some cultivars may overall look stunted (e). Distinctive downward bending of the affected leaves may also be observed (f, g).

4.5 TaVCV – description

4.5.1 Particle Morphology

Thin section electron microscopy analysis shows that TaVCV virions are typically bullet shaped and measure approximately 200 x 70 nm in size (Revill et al., 2005a) (Fig. 1.3).

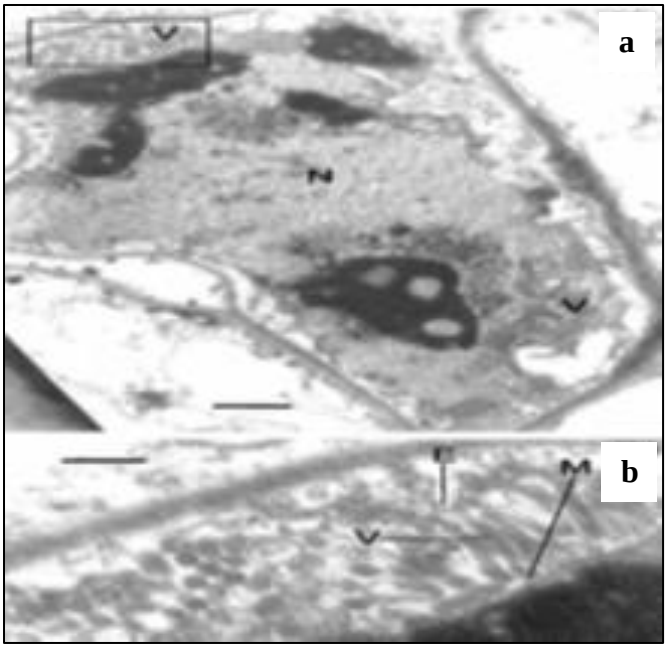


Fig. 1.3. Electron micrographs of cross-sections through a TaVCV-infected cell (a) showing the nucleus (N) containing TaVCV virions (V). Bar: 500 nm. The boxed area is enlarged in (b) and shows TaVCV virions (V) in the perinuclear space between the inner membrane (M) and outer membrane (E) of the nuclear envelope. Bar: 200 nm. Adopted from Revill et al. (2005a).

4.5.2 Genome Organization

The negative sense ssRNA TaVCV genome is comprised of 12,020 nucleotides and contains six major ORFs. In agreement with other sequenced members of the *Rhabdoviridae* family, the TaVCV genome organization is 3'-leader-N-P-3-M-G-L-trailer-5' (Fig. 1.4). The length of these six ORFs are: 1506 (N), 813 (P), 861 (3), 705 (M), 1764 (G) and 5784 (L) nucleotides (Revill et al., 2005a). The function of gene 3 remains to be validated, although it is present at the same position as the sc4 gene of SYNV and gene 3 of MMV and RYSV. sc4 has been shown to be a putative movement protein (MP) in SYNV (Goodin et al. 2002; Scholthof et al. 1994). Likewise, gene 3 has a MP function in RYSV (Huang et al. 2005).

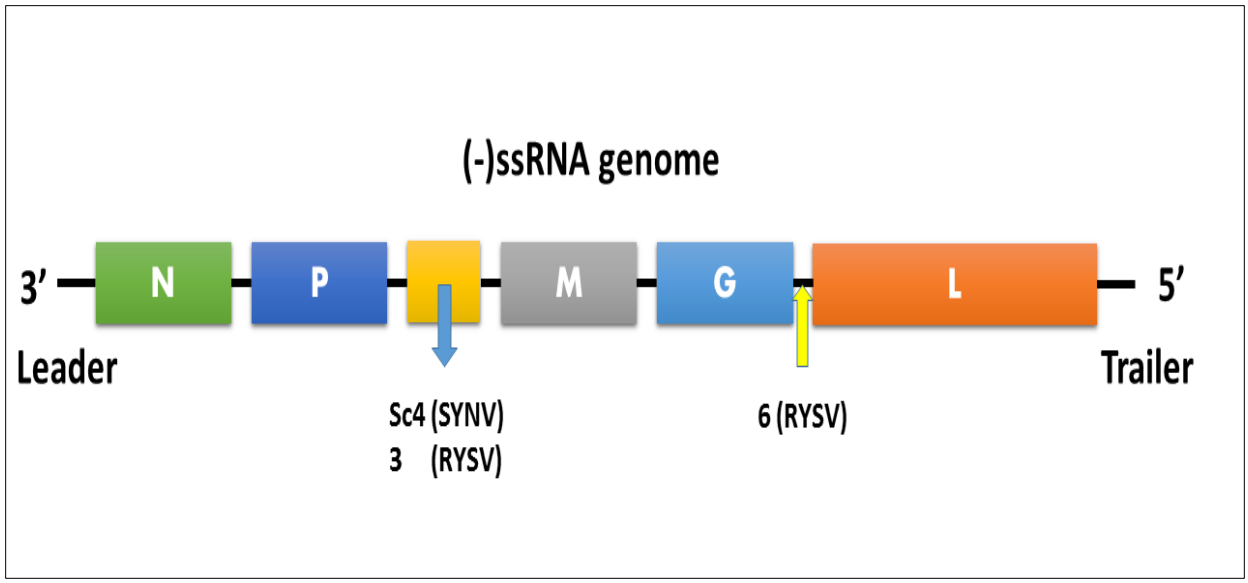


Fig. 1.4. Genome arrangement of TaVCV, in coding orientation. N, Putative nucleocapsid gene; P, putative phosphoprotein gene; 3, gene 3; M, putative matrix protein gene; G, glycoprotein gene; L, polymerase protein gene. Adopted from Revill et al. (2005a).

4.5.3 Transmission

The vector for TaVCV is not known. The closely related CBDaV, another member of the *Rhabdoviridae* family is vectored by the taro planthopper *Tarophagus proserpina* (family Delphacidae) (Gollifer et al., 1977). Other planthopper transmitted viruses of the genus *Nucleorhabdovirus* include MMV which is vectored by the corn planthopper *Peregrinus maidis* and *Maize Iranian mosaic virus* (MIMV) vectored by the planthopper *Ribautodelphax notabilis* (ICTV, online). Leafhoppers (family Cicadellidae) are also prominent vectors of this genus. MFSV is transmitted by the blackfaced leafhopper *Graminella nigrifrons* (Redinbaugh et al., 2002; Todd et al. 2010) while RYSV is transmitted by green rice leafhoppers *Nephotettix nigropictus, N. cincticeps,* and *N. virescens* (Chiu et al., 1965; Hiraguri et al., 2014). PYDV is transmitted by clover leafhoppers of the species *Aceratagallia sanguinolenta* (Black, 1943)

whereas EMDV is vectored by the Agalliinae leafhoppers *Anaceratogallia laevis*, *A. ribauti* and *Agallia vorobjevi* (Babaie & Izadpanah, 2003), Sorghum stunt mosaic virus (SSMV) by the lesser lawn leafhopper *Graminella sonora* (Mayhew & Flock, 1981; Creamer, 1992) and *Wheat American striate mosaic virus* (WASMV), a *Cytorhabdovirus*, by the painted leafhopper *Endria inimica* and blackfaced leafhopper *Graminella nigrifrons* (ICTV, online).

Only two out of the ten *Nucleorhabdovirus* species are vectored by aphids. These include the *Sonchus yellow net virus* (SYNV) transmitted by *Aphis coreopsidis* (Christie, Christie, & Edwardson, 1974) and *Sowthistle yellow vein virus* (SYVV) transmitted by the blackcurrant-sowthistle aphid *Hyperomyzus lactucae* (Duffus, 1963; Richardson & Sylvester, 1968) and somewhat inefficiently by the potato aphid *Macrosiphum euphorbiae* (Behncken, 1973). TaVCV is unlikely to be spread by seed or pollen. Conversely, it is certainly spread through the use of infected planting material (Harding, n.d.; Long et al., 2014; Revill et al., 2005b). Based on these relationships, TaVCV is most likely vectored by a planthopper or leafhopper. Three species of taro planthoppers have been described; *Tarophagus colocasiae*, *T. persephone* and *T. proserpina* with varying distribution around Asia and the Pacific (Asche & Wilson, 1989). However, any association cannot be made of these planthoppers with TaVCV transmission unless studies are clearly able to demonstrate a relationship.

CHAPTER 2: GENETIC DIVERSITY AMONG HAWAIIAN AND PALAUAN *TARO VEIN CHLOROSIS VIRUS* (TAVCV) ISOLATES

Introduction

Plant viruses with RNA genomes are more susceptible to genetic variation within the greater plant virus group and this in turn has facilitated their continued survival in changing environments and new or previously resistant hosts (Holmes, 2009). The former is also partly due to large population sizes of RNA viruses, short generation time and high mutation rates as a result of error-prone replication because their RNA-dependent RNA polymerases (RdRp genes) lack proofreading activity (Domingo & Holland, 1997). Additionally, studies have provided evidence that plant RNA viruses are more frequently prone to recombination events with closely or distantly related viruses, even with host genomes and this creates selection forces that considerably influence their genome evolution and divergence (Sztuba-Solin'ska et al., 2011).

Significant genetic diversity has been reported in field isolates of two members of the genus *Cytorhabdovirus; Lettuce necrotic yellows virus* (LNYV) (Callaghan & Dietzgen, 2005; Higgins et al., 2016) and *Strawberry crinkle virus* (SCV) (Klerks et al., 2004) and *Taro vein chlorosis virus* (TaVCV) (Revill et al., 2005a; Jackson et al., 2005), a *Nucleorhabdovirus* with anti-sense RNA genome and the subject of the present study. TaVCV was first reported in Hawaii in 2013 (Long et al., 2014), however, no further studies with the virus were conducted thereafter. Taking the above cases into consideration and the fact that limited knowledge existed about TaVCV in Hawaii, a diversity study was undertaken. This chapter is a continuation of work from the initial survey for TaVCV on five of the major Hawaiian Islands; Big Island (Hawaii), Kauai, Maui, Molokai and Oahu by Long et al. (2014) as well as samples from Palau.

Materials and Methods

Sample collection

Hawaii: Entire leaf samples from 328 plants with putative viral symptoms as well as from healthy controls were collected from 9 locations on Big Island (Hawaii), 8 locations on Kauai, 7 locations on Maui, 3 locations on Molokai and 8 locations on Oahu between summer of 2012 and summer of 2013 (Table 2.1). These locations comprised of commercial taro farms, University of Hawaii taro germplasm collections, botanical gardens and naturalized plants. All leaf samples were kept on ice during collection and stored at -20°C in the laboratory until processed. All samples were documented, photographed and linked to a photo-database. All of the 328 samples were tested and the results were published in the first report of TaVCV from Hawaii and the USA by Long et al. (2014). Only the TaVCV positive samples from the initial survey were used for this study.

Palau: Leaf tissue from symptomatic as well as healthy looking plants were collected from the state of Airai, located on the southern coast of Babeldaob island in spring of 2016. These samples were kindly provided by Dr. Shizu Watanabe (University of Hawaii).

RNA isolation and cDNA synthesis

For all samples, apart from the Palauan taro leaf samples, total RNA was isolated from 100 mg of leaf tissue using a NucleoSpin® RNA II kit (Macherey-Nagel, Bethlehem, PA) following the manufacturers protocol. For the Palauan samples, total RNA was extracted using Plant RNeasy Mini Kit (Qiagen® Inc., Valencia, CA) following the manufacturer's protocol. RNA eluted in 50 µl of sterile H_2O was capped with 50 µl 95% ethanol, stored at -20°C and brought to Hawaii for analysis. RNA was recovered by precipitation: 100 µl 95% ethanol and 20

µl (0.1v) 3M sodium acetate was added per sample and left to incubate overnight. The next day, each sample was centrifuged at maximum speed for 20 mins at 4°C. The RNA pellet was re-suspended in 20 µl nuclease free, sterile H_2O after the supernatant was removed.

First strand cDNA copies of TaVCV RNA were generated using random primer (RP) 470 (5'-GCC GGA GCT CTG CAG AAT TCN NNN NN-3') and the MMLV reverse transcriptase system (Promega, Madison, WI): for each sample, 2 µl extracted RNA was added to 6.5 µl RNase/nuclease free H_2O and 10 pmol µl RP 470, heat denatured at 70°C for 8 – 10 minutes then quickly chilled on ice. Then 5 µl of 2mM dNTP mix, 4 µl reaction buffer, 1 µl RT (MMLV) and 0.5 µl rRNasin® were added for a final volume of 20 µl. The reaction was incubated at 25°C for 5 mins and then at 42°C for 55 minutes.

RT-PCR

cDNA was used as template in a 20 µl standard PCR: for each sample, 1 µl cDNA was added to 10 µl 2 x GoTaq Green Master Mix (Promega, Madison, WI), 7 µl ddH_2O and 1 µl each (10 µM concentration) of forward and reverse virus-specific primers Pol2A1 (5'-AAT ATG CTC TCC AGT GTT CAC CC-3') and Pol2A2 (5'-AGG TGC TCA AAT GAC TCA GCT TGT CC-3') (Revill et al. 2005a). This primer set targeted a 952 bp region in the RdRp gene of the TaVCV genome. The cycling parameters were as follows: initial denaturing step at 95°C for 5 minutes and then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. Amplicons were separated by electrophoresis through a 1% (w/v) TAE/Agarose gel stained with ethidium bromide dye. TaVCV bands (952 bp) were confirmed against the Gene Ruler DNA ladder (Thermo Fisher Scientific) over a UV

Transilluminator (Labnet Inc., Edison, NJ), photographed with an 'ethidium bromide filter' camera (FOTODYNE®, Hartland, WI) and printed for comparative analysis.

Molecular cloning and sequencing

PCR products were collected by excising the 952 bp fragment from the agarose gel followed by centrifugation at maximum speed for 2 minutes in a 'double microfuge system' (inner smaller 0.5 ml microfuge tube punctured at the bottom, carefully lined with GF/C filter paper (Whatman, Pittsburgh, PA) and fitted into a 1.5 ml microfuge tube). Either 3.5 µl of this elute or 1 µl PCR product was directly ligated into 0.5 µl pGEM-T Easy cloning vector (Promega) in a 10 µl reaction containing 5 µl 2 x Ligation buffer, 1 µl T4 DNA Ligase and 2.5 µl distilled water following the manufacturer's protocol.

The ligated plasmids were incorporated into *Escherichia coli* (DH5α) cells by first incubating 5 µl of the ligation product with 100 µl of homemade competent *E. coli* on ice for 60 minutes, heat-shocking the reaction at 42°C for 45 seconds then adding 400 µl SOB medium and 2 µl of 2M $MgCl_2$ followed by a further incubation at 37°C for 45 minutes. The reaction was plated on McConkey agar/ampicillin plates and incubated at 37°C overnight.

Transformed colonies appeared pale-colorless, that is, did not develop a reddish-pink hue on the medium allowing visual selection. The selected colonies were cultured in 2 ml LB broth overnight then purified using the Mini-Prep Lab – QIA prep spin protocol (Qiagen® Inc., Valencia, CA) following the manufacturer's guidelines. Samples were sequenced at the Greenwood Biotech Core Laboratory or the Advanced Studies in Genomics, Proteomics and Bioinformatics Laboratory using 3.2 pmol SP6 (5'-TAC GAT TTA GGT GAC ACT ATA G-3') and T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') primers in an 8 µl sequence reaction.

Sequence assembly

Sequences were analyzed using the blastn application on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Each sequence was confirmed in at least three clones for every sample in both forward and reverse orientations. Contiguous sequences were generated using the Cap 3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3). The same online program was also used to assemble consensus sequences for each isolate. The consensus nucleotide sequences were then translated into protein sequences at the ExPASy website (http://web.expasy.org/translate/).

Assessing the genetic diversity of TaVCV

The RdRp gene sequence data was used to evaluate the genetic diversity of the Hawaiian and Palauan TaVCV population. The primer sequences of Pol2A1 (F) and Pol2A2 (R) used to amplify the RdRp gene were trimmed from this dataset in subsequent analyses using the Jalview software available at http://www.jalview.org/. Estimates of genetic diversity were generated using DnaSP 5.10.1 (Rozas, 2009). Three models were used for this analysis: Jukes-Cantor test, Fu & Li's Test, and Tajima's Test. Additionally, 20 randomly chosen nucleotide and deduced amino acid sequences were subjected to LALIGN analysis (Huang & Miller, 1991); this program is also available online at http://embnet.vital-it.ch/software/LALIGN_form.html.

Phylogenetic analyses

Both the nucleotide and deduced amino acid sequences were aligned using the ClustalX 2.1 program (Larkin et al., 2007). Neighbor-joined phylogenetic trees were constructed and Maximum Parsimony and Maximum Likelihood analyses were conducted with the Seaview

software (Gouy, Guindon, & Gascuel, 2010). Sequence information for the partial L (RdRp) gene for a Molokai isolate and a fully characterized TaVCV isolate from Fiji used in all phylogenetic analyses were obtained from GenBank; accessions KF921086 and AY674964 respectively. Two closely related nucleorhabdoviruses, *Maize mosaic virus* (MMV) (GenBank accession NC_005975) and *Maize Iranian mosaic virus* (MIMV) (GenBank accession NC_011542) were also included in all phylogenetic assessments.

Results

Incidence of TaVCV in Hawaii and Palau samples

Out of the 71 positive samples from the initial 2012/2013 Hawaii survey, 65 were positive for TaVCV (Table 2.1) representing 19.8% of the 328 plants sampled; 91.5% of the original number of positives. TaVCV positive samples were obtained from each of the 5 islands with highest disease incidence recorded in Molokai (44.2%, n = 52), followed by Kauai (26.5%, n = 68), Maui (25.4%, n = 71), Big Island (5.1%, n = 79) and Oahu (3.4%, n = 58).

Three out of the eight samples (37.5%) from Palau were TaVCV positive (Table 2.1).

Table 2.1. Summary of the TaVCV survey conducted in Hawaii and TaVCV test for samples received from Palau. The first percentage value in brackets indicates the ratio of positives versus the total number of samples collected per island. The second percentage value in brackets indicates the ratio of positives per island against the collective total number of samples for the State. The values in parenthesis show the number of positive samples obtained in 2014 using the same primer set (Pol2A1/Pol2A2) and protocol compared to the initial survey in 2012/2013.

Island	**Location #**	**Samples collected**	**Number of TaVCV positive samples**
Big Island (Hawaii)	1	17	2
	2	6	0
	3	2	0
	4	7	1
	5	5	0
	6	12	0
	7	3	0
	8	4	0
	9	23	4
		79	***7 (8.9%, 2.1%) [4]***
Kauai	1	8	0
	2	14	6
	3	8	1
	4	3	0
	5	2	0
	6	2	0
	7	8	7
	8	23	6
		68	***20 (29.4%, 6.1%) [18]***

Table 2.1. (Continued) Summary of the TaVCV survey conducted in Hawaii and TaVCV test for samples received from Palau.

Maui	1	3	0
	2	2	0
	3	5	0
	4	13	5
	5	14	4
	6	11	1
	7	23	9
		71	***19 (26.8%, 5.8%) [18]***
Molokai	1	28	15
	2	13	7
	3	11	1
		52	***23 (44.2%, 7.0%) [*23]***
Oahu	1	2	0
	2	12	0
	3	16	0
	4	5	0
	5	1	0
	6	9	2
	7	6	0
	8	7	0
		58	***2 (3.4%, 0.6%) [2]***
Grand Total	***35***	***328***	***71 (21.6%) [65]***
Palau	**1**	**8**	**3 (37.5%)**

*None of the 23 Molokai isolates could be sequenced as part of this study.

Genetic diversity amongst Hawaiian and Palauan TaVCV

Approximately 1 kb (952 bp) of the L (RdRp) gene sequences of TaVCV were used to assess its diversity in Hawaii and also to compare variability versus the 3 Palauan and 1 Fijian isolate; the latter obtained from GenBank (accession AY674964). From the 65 TaVCV positive samples for the Hawaii, 42 were successfully sequenced: Kauai = 18, Maui =18, Big Island (Hawaii) = 4 and Oahu = 2. All the 3 positive samples from Palau were also sequenced. Sequence for the same gene for a Molokai isolate was obtained from GenBank (accession KF921086).

Twenty sequences were randomly selected from these 47 sequences and subjected to LALIGN analysis. The Hawaiian isolates were 98.2 to 99.9% identical at the nucleotide level and 97.4 to 100% identical at the translated amino acid level when compared against each other. The same pool of sequences were 97.7 to 98.8% identical at the genomic level and 97.7 – 99.0% identical at the deduced amino acid level versus Palauan sequences (Table 2.3).

On the other hand, the Palauan sequences were strikingly 99.9 – 100% identical at the nucleotide level and 100% identical at the amino acid level amongst each other. Interestingly, a variance of 21 – 22% at the nucleotide level and 6.5 – 8.7% at the protein level were noted when the Hawaiian and Palauan partial RdRp sequences were compared against the Fijian sequence (Table 2.3). The low genetic diversity values amongst Hawaiian and Palauan isolates obtained in the LALIGN analysis were supported by DnaSP 5.10.1. Of the 952 nucleotide positions of the RdRp gene analyzed, 92 (9.7%) were polymorphic.

The nucleotide diversity values of the RdRp gene ranged from 0.00532 to 0.00780 [π(JC)] for the different islands averaging out to 0.00703 for the State (Table 2.2). The Pi (π) values of the Jukes-Cantor calculation for nucleotide diversity were in substantial agreement

with Fu & Li's calculation as well as Tajima's assessment. Additionally, Fu & Li's D test, F test and Tajima's D test values were all in negatives and not statistically significant, hence, not in favor of a genetically diverse population.

The sequences for the 3 Palauan isolates were tremendously similar with each other and had a nucleotide diversity value of 0.00070 (Table 2.2). The overall assessment was reinforced by phylogenetic analysis of the translation of these sequences. All sequences from the Hawaii population grouped closely in one clade while the Palauan sequences formed a separate clade. The Fijian isolate grouped into a third clade alone while MMV and MIMV sequences branched as outgroups (Fig 2.2).

Table 2.2. Nucleotide diversity of the *Taro vein chlorosis virus* in Hawaiian and Palauan isolates. Nucleotide diversity values calculated using Jukes-Cantor, Fu & Li's and Tajima's methods are displayed in columns 3, 4 and 7 respectively. Fu & Li's D and F tests and Tajima's D test values for statistical significance are shown in columns 5, 6 and 8. The analyzed region is 952 nt long in the RdRp gene.

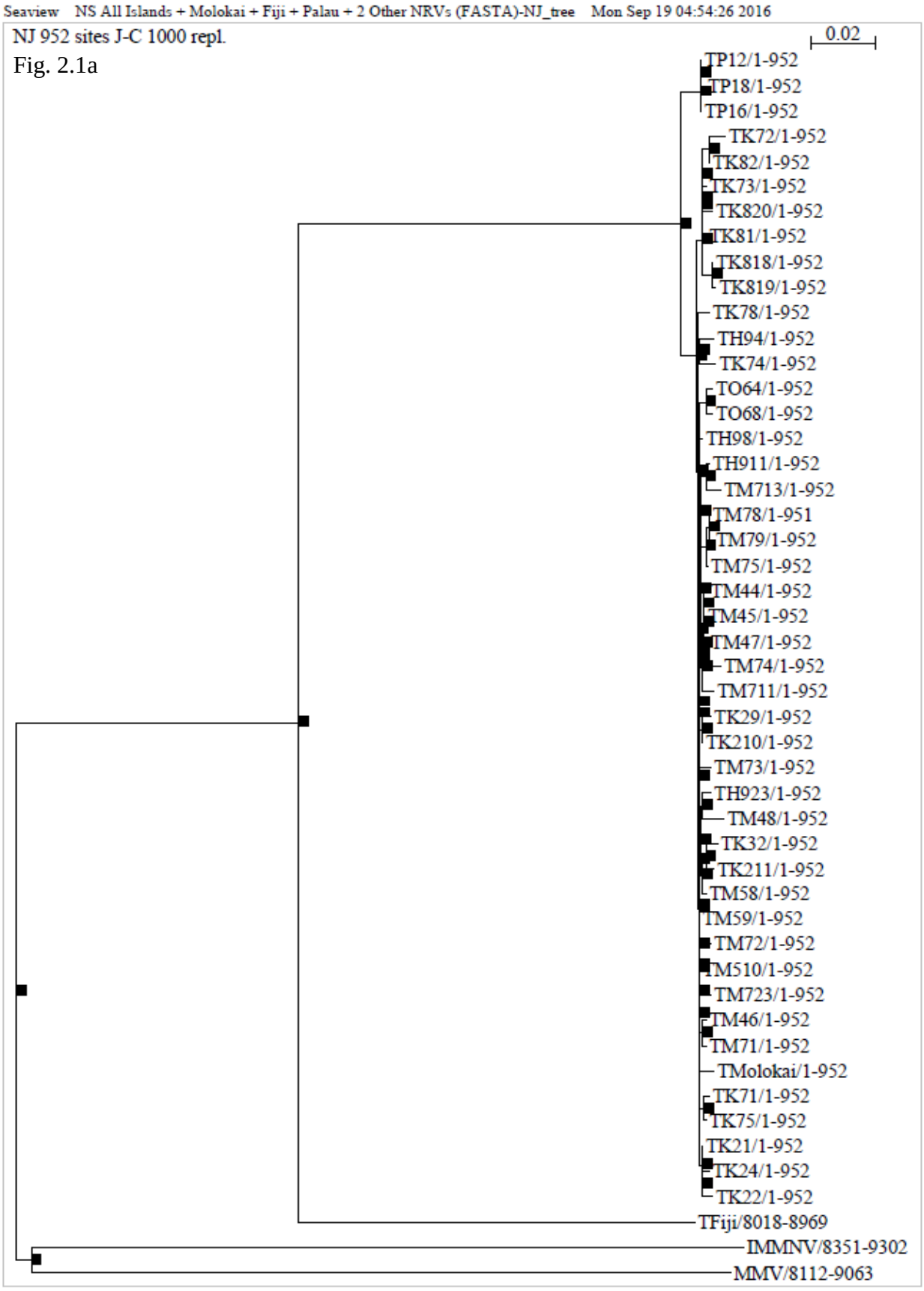
Location	**n**	**π(JC)**	**π(FL)**	**D Test(FL)**	**F Test(FL)**	**π(T)**	**D Test(T)**
Kauai	18	0.00780	0.00776	-2.33459	-2.5242	0.00776	-1.78593
Maui	18	0.00532	0.00529	-2.92604	-3.17255	0.00529	-2.26604
Hawaii	4	0.00633	0.00630	-0.84046	-0.84986	0.00630	-0.84046
Oahu	2	0.00421	*	*	*	*	*
Molokai	1	*	*	*	*	*	*
State	**43**	**0.00703**	**0.00699**	**-4.10777**	**-4.22551**	**0.00699**	**-2.54752**
Palau	3	0.00070	*	*	*	*	*

* Either two or four and more sequences were required to compute these values

Phylogenetic analyses

The partial L genomes and proteomes of 43 Hawaiian TaVCV isolates and 3 Palauan TaVCV isolates were analyzed to elucidate their phylogenetic relationships. Two isolates from the Hawaiian island of Maui, TM713 and TM723, were omitted from phylogenetic analyses due to anomalies in their data. These sequences, when translated into amino acid sequences are truncated by 65 amino acids towards the 5' end, which appear to be due to sequencing and/or translation errors.

Neighbor joined, Maximum Parsimony and Maximum Likelihood trees were constructed. Analyses using the nucleotide and deduced amino acid sequences, regardless of the software or online resource being used, produced nearly identical results (Fig. 2.1, 2.2). In both the nucleotide and protein trees, all the Hawaiian isolates closely grouped together in one clade. The 3 Palauan isolates formed a second clade. The Fijian isolate separated into a third clade while MMV and MIMV sequences clearly formed outgroups (Fig. 2.1, 2.2).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0.02
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TP18/1-952
TP16/1-952
TK72/1-952
TK82/1-952
TK73/1-952
TK820/1-952
TK81/1-952
TK818/1-952
TK819/1-952
TK78/1-952
TH94/1-952
TK74/1-952
TO64/1-952
TO68/1-952
TH98/1-952
TH911/1-952
TM713/1-952
TM78/1-951
TM79/1-952
TM75/1-952
TM44/1-952
TM45/1-952
TM47/1-952
TM74/1-952
TM711/1-952
TK29/1-952
TK210/1-952
TM73/1-952
TH923/1-952
TM48/1-952
TK32/1-952
TK211/1-952
TM58/1-952
TM59/1-952
TM72/1-952
TM510/1-952
TM723/1-952
TM46/1-952
TM71/1-952
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TK75/1-952
TK21/1-952
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TK22/1-952
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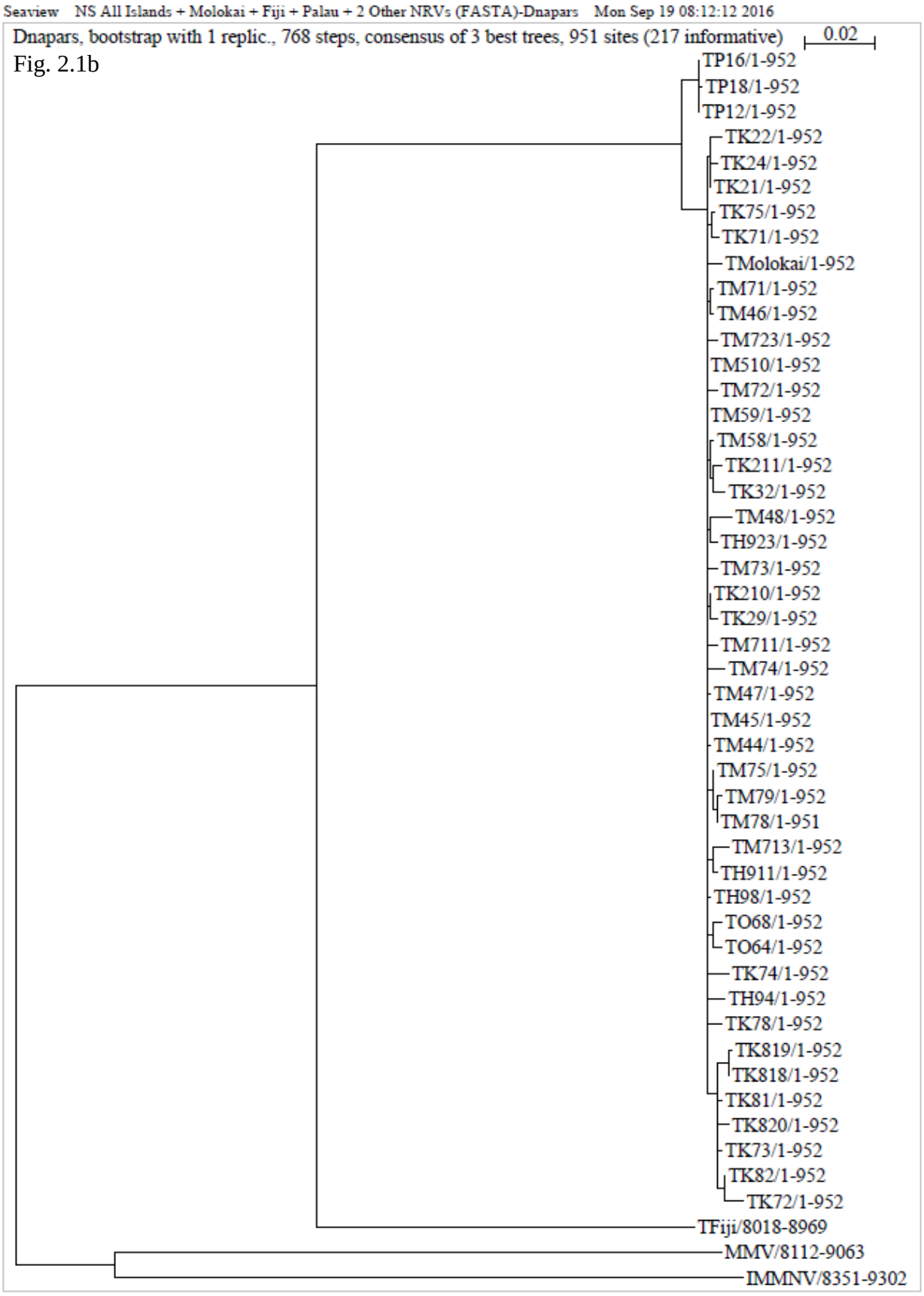
Fig. 2.1a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Dnapars, bootstrap with 1 replic., 768 steps, consensus of 3 best trees, 951 sites (217 informative)
0.02
TP16/1-952
TP18/1-952
TP12/1-952
TK22/1-952
TK24/1-952
TK21/1-952
TK75/1-952
TK71/1-952
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TM71/1-952
TM46/1-952
TM723/1-952
TM510/1-952
TM72/1-952
TM59/1-952
TM58/1-952
TK211/1-952
TK32/1-952
TM48/1-952
TH923/1-952
TM73/1-952
TK210/1-952
TK29/1-952
TM711/1-952
TM74/1-952
TM47/1-952
TM45/1-952
TM44/1-952
TM75/1-952
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TH94/1-952
TK78/1-952
TK819/1-952
TK818/1-952
TK81/1-952
TK820/1-952
TK73/1-952
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Fig. 2.1b

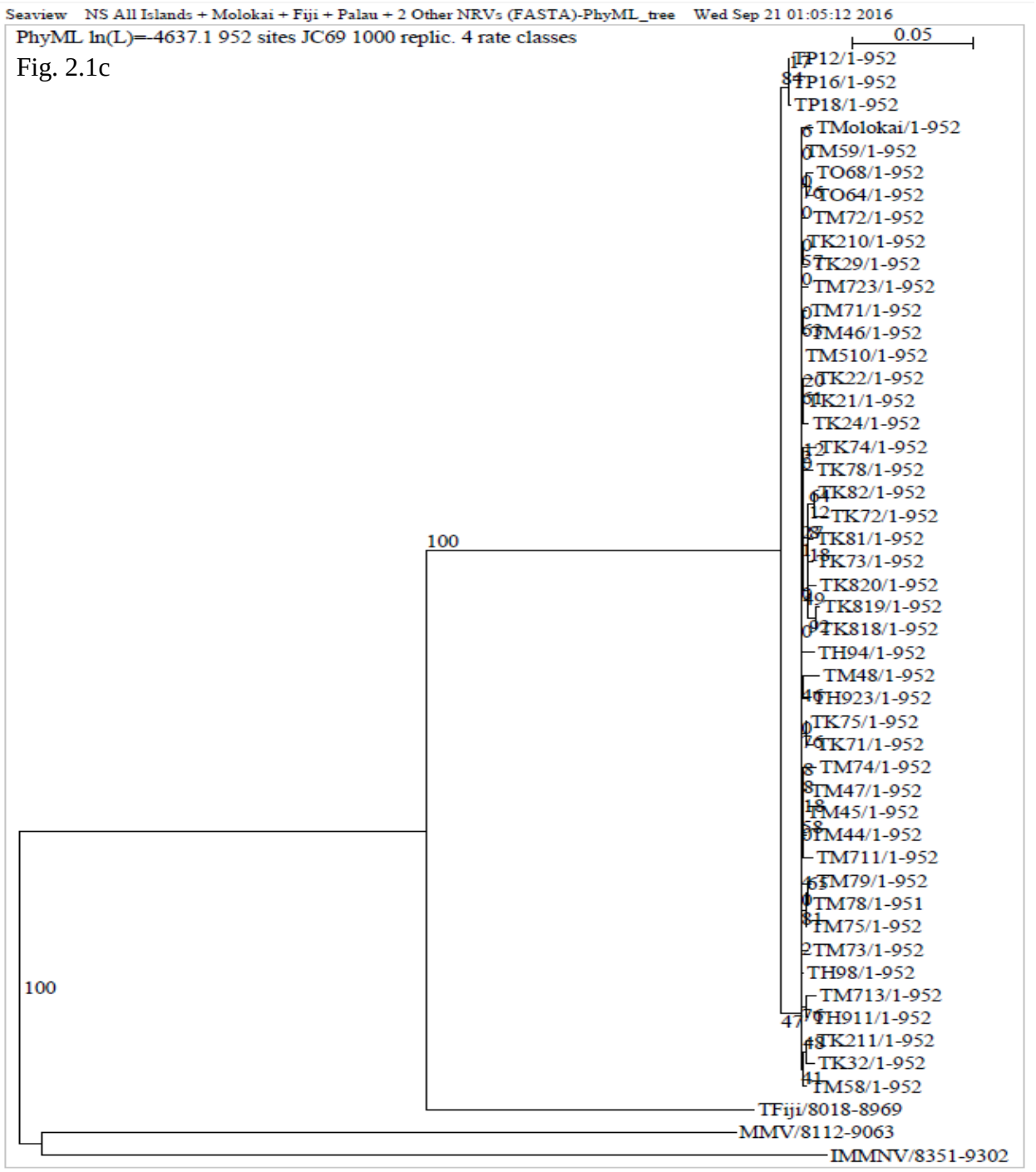
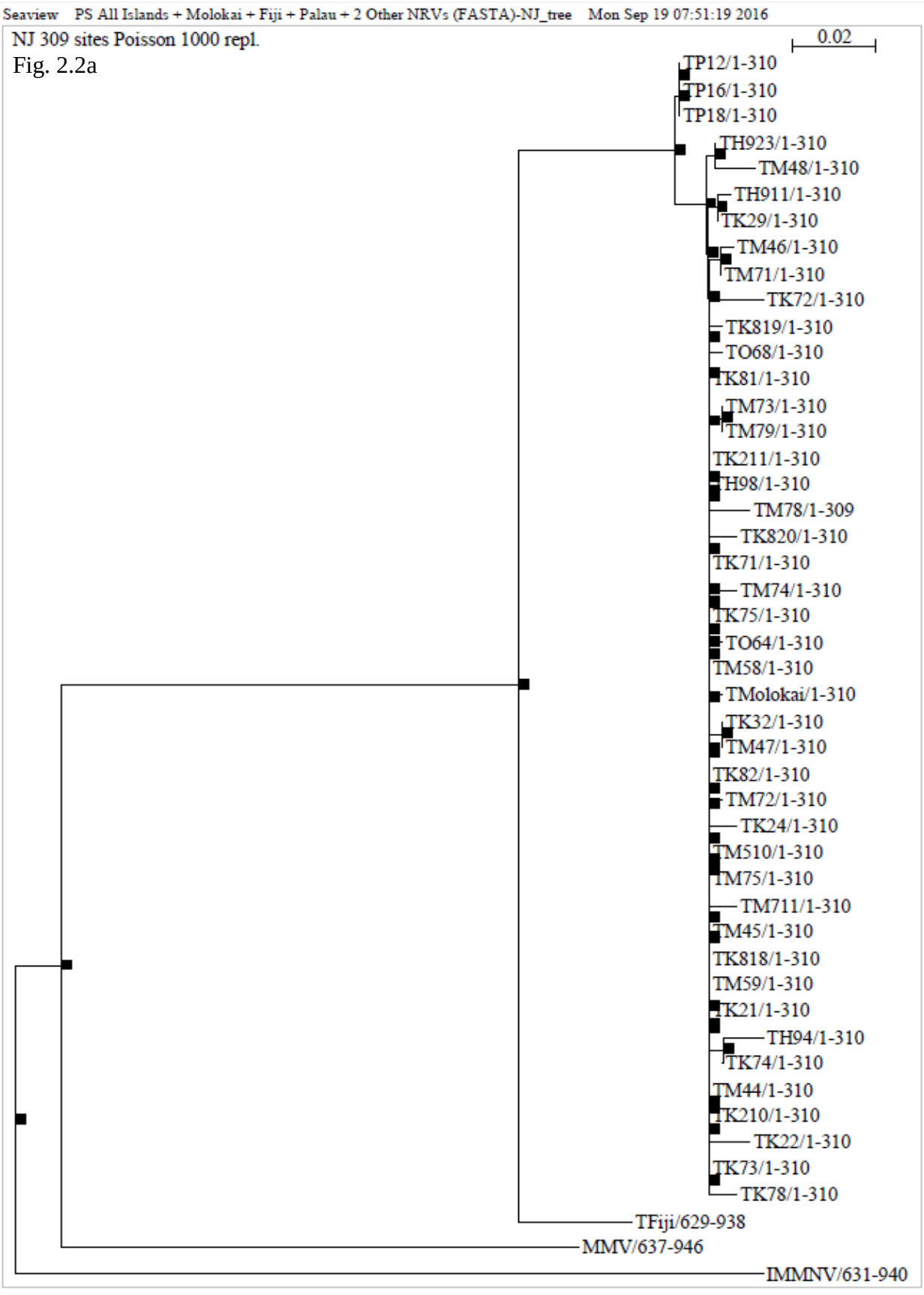


Fig. 2.1a – c. Neighbor joined (a), Maximum Parsimony (b) and Maximum Likelihood (c) phylogenetic trees of the partial TaVCV L (RdRp) genome of Hawaiian and Palauan isolates compared with partial polymerase sequences of a Fijian TaVCV isolate and 2 closely related Nucleorhabdoviruses – MMV and IMMV. Bootstrap values (from 1000 replicates) are shown at select nodes.


Seaview PS All Islands + Molokai + Fiji + Palau + 2 Other NRVs (FASTA)-NJ_tree Mon Sep 19 07:51:19 2016
NJ 309 sites Poisson 1000 repl.
Fig. 2.2a
0.02
TP12/1-310
TP16/1-310
TP18/1-310
TH923/1-310
TM48/1-310
TH911/1-310
TK29/1-310
TM46/1-310
TM71/1-310
TK72/1-310
TK819/1-310
TO68/1-310
TK81/1-310
TM73/1-310
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TH98/1-310
TM78/1-309
TK820/1-310
TK71/1-310
TM74/1-310
TK75/1-310
TO64/1-310
TM58/1-310
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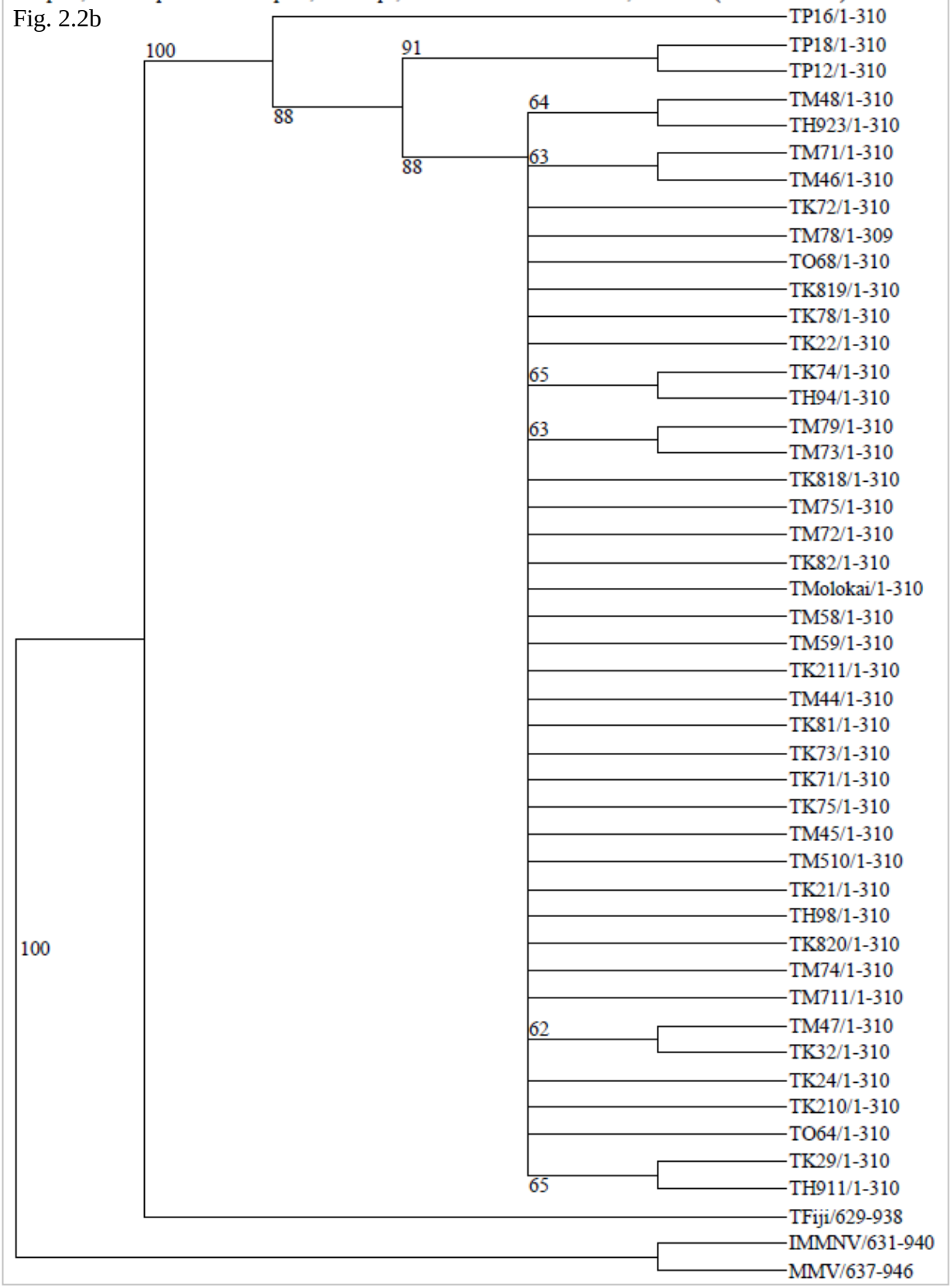
TM47/1-310
TK82/1-310
TM72/1-310
TK24/1-310
TM510/1-310
TM75/1-310
TM711/1-310
TM45/1-310
TK818/1-310
TM59/1-310
TK21/1-310
TH94/1-310
TK74/1-310
TM44/1-310
TK210/1-310
TK22/1-310
TK73/1-310
TK78/1-310
TFiji/629-938
MMV/637-946
IMMNV/631-940

Seaview PS All Islands + Molokai + Fiji + Palau + 2 Other NRVs (FASTA)-Protpars Sat Sep 24 21:55:19 2016

Protpars, bootstrap with 1000 replic., 231 steps, consensus of 100 best trees, 309 sites (36 informative)

Fig. 2.2b



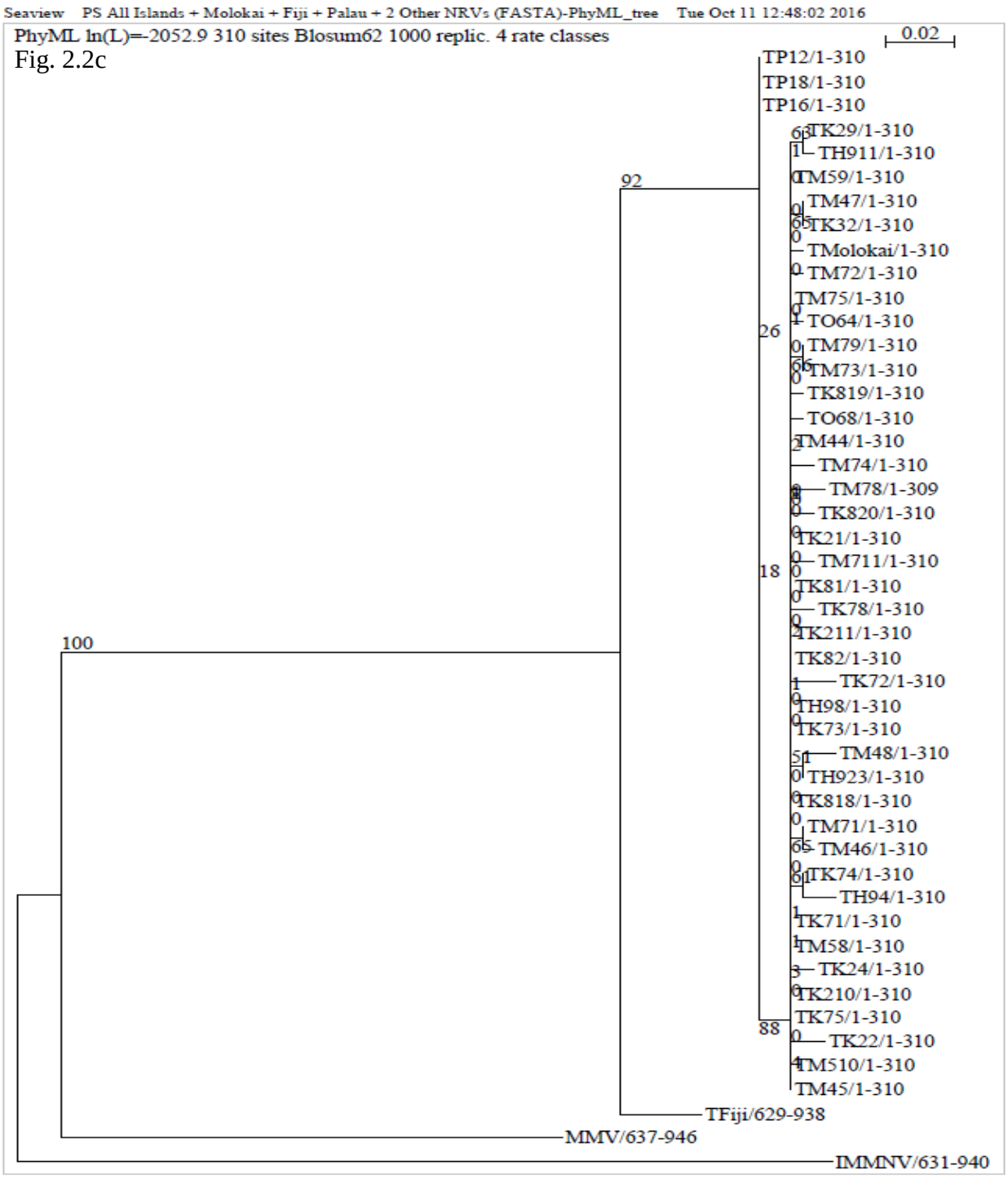


Fig. 2.2a – c. Neighbor joined (a), Maximum Parsimony (b) and Maximum Likelihood (c) phylogenetic trees of the partial TaVCV L gene deduced amino acid sequences of Hawaiian and Palauan isolates compared with partial polymerase protein sequences of a Fijian TaVCV isolate and 2 closely related Nucleorhabdoviruses – MMV and IMMV. Bootstrap values (from 1000 replicates) are shown at select nodes.

Table 2.3. Nucleotide (upper right) and amino acid (lower left) percent identity comparisons between partially sequenced isolates of *Taro vein chlorosis virus* (TaVCV). Nucleotide comparisons are based upon approximately 1 kb (952 bp) region of the L (RdRp) sequences translated into peptide sequences with 310 amino acids. Partial genome sequence of the Molokai isolate (TMol) and full genome sequence of the Fijian isolate (TFiji) were obtained from GenBank (accession numbers accessions KF921086 and AY674964 respectively). TP12, TP16 and TP18 represent the 3 isolates sequenced from Palau.

Nucleotide identities (%)

Amino acid identities (%)

	TK72	TM48	TH94	TK819	TH911	TO68	TM74	TM72	TO64	TH923	TK24	TM59	TK210	TM44	TK32	TMol	TP12	TP16	TP18	TFiji
TK72		98.2	98.4	98.8	98.6	98.5	98.3	98.6	98.5	98.6	98.6	98.9	98.8	98.7	98.4	98.5	97.8	97.8	97.7	78.6
TM48	97.4		98.7	98.5	98.9	98.8	98.7	98.9	98.8	99.2	98.9	99.3	99.2	99.1	98.7	98.7	98.1	98.1	98	78.0
TH94	97.4	97.4		98.7	99.2	99.1	98.8	99.2	99.1	99.2	99.2	99.5	99.4	99.3	98.9	99.1	98.3	98.3	98.2	78.4
TK819	98.4	98.4	98.4		98.9	98.8	98.6	98.9	98.8	98.9	98.9	99.3	99.2	99.1	99.2	98.8	98.3	98.3	98.2	78.4
TH911	98.1	98.4	98.1	99.0		99.3	99.1	99.4	99.3	99.4	99.4	99.7	99.6	99.5	99.2	99.3	98.5	98.5	98.4	78.4
TO68	99.7	98.4	98.4	99.4	99.0		98.9	99.3	99.6	99.3	99.3	99.6	99.5	99.4	99.1	99.2	98.4	98.4	98.3	78.6
TM74	98.1	98.1	98.1	99.0	98.7	99.0		99.1	98.9	99.1	99.1	99.4	99.3	99.4	98.8	98.9	98.2	98.2	98.1	78.3
TM72	98.a	98.4	98.4	99.4	99.0	99.4	99.0		99.3	99.4	99.4	99.7	99.6	99.5	99.2	99.3	98.5	98.5	98.4	78.4
TO64	98.4	98.4	98.4	99.4	99.0	99.4	99.0	99.4		99.3	99.3	99.6	99.5	99.4	99.1	99.2	98.4	98.4	98.3	78.8
TH923	98.4	99.0	98.4	99.4	99.0	99.4	99.0	99.4	99.4		99.4	99.7	99.6	99.5	99.2	99.3	98.5	98.5	98.4	78.4
TK24	98.1	98.1	98.1	99.0	98.7	99.0	98.7	99.0	99.0	99.0		99.7	99.6	99.5	99.2	99.3	98.5	98.5	98.4	78.6
TM59	98.7	98.7	98.7	99.7	99.4	99.7	99.4	99.7	99.7	99.7	99.4		99.9	99.8	99.5	99.6	98.8	98.8	98.7	78.7
TK210	98.7	98.7	98.7	99.7	99.4	99.7	99.4	99.7	99.7	99.7	99.4	100		99.7	99.4	99.5	98.7	98.7	98.6	78.6
TM44	98.7	98.7	98.7	99.7	99.4	99.7	99.4	99.7	99.7	99.7	99.4	100	100		99.3	99.4	98.6	98.6	98.5	78.5
TK32	98.4	98.4	98.4	99.4	99.0	99.4	99.0	99.4	99.4	99.4	99.0	99.7	99.7	99.7		99.1	98.5	98.5	98.4	78.4
TMol	98.4	98.4	98.4	99.4	99.0	99.4	99.0	99.4	99.4	99.4	99.0	99.7	99.7	99.7	99.4		98.4	98.4	98.3	78.9
TP12	97.7	97.7	97.7	98.7	98.4	98.7	98.4	98.7	98.7	98.7	98.4	99.0	99.0	99.0	98.7	98.7		100	99.9	79.1
TP16	97.7	97.7	97.7	98.7	98.4	98.7	98.4	98.7	98.7	98.7	98.4	99.0	99.0	99.0	98.7	98.7	100		99.9	79.1
TP18	97.7	97.7	97.7	98.7	98.4	98.7	98.4	98.7	98.7	98.7	98.4	99.0	99.0	99.0	98.7	98.7	100	100		79
TFiji	91.6	91.3	91.3	92.3	91.9	92.3	91.9	92.3	92.3	92.3	91.9	92.6	92.6	92.6	92.3	92.3	93.5	93.5	93.5	

Discussion

Taro is an important staple for Hawaii and most of the Pacific island countries. It is also culturally invaluable to the *'Kanaka maoli'* (Hawaii's indigenous people) and therefore recognized as the state plant (McPherson, 2013). TaVCV is the newest addition to the list of pests and pathogens affecting this crop. Although the discovery of the virus is very recent, it has been found affecting taro throughout the state (Long et al., 2014). In this study, 65 positive samples were identified by RT-PCR amplification of the partial L gene of TaVCV from 328 plants sampled.

This number was six less than the total number of positives (71) identified when the samples were initially tested in 2012/2013 by Long et al. (2014). Understandably, the RNA and/or cDNA for these samples may have degraded over long term storage although they were kept at -80°C. Twenty-three samples from Molokai appeared to be TaVCV positive, however, their PCR bands were very faint and none of these could be successfully cloned and sequenced. TaVCV has previously been reported from Palau (Revill et al., 2005b; Harding, 2008) yet no diversity studies have been conducted. Hence, the eight samples received from Palau were of particular interest. Three samples tested positive and were subsequently sequenced.

Very little nucleotide sequence variation, 0.1 – 1.8%, were observed within the Hawaiian TaVCV isolates. Their translated amino acid sequences had 0 – 2.6% variation. Because positive samples were obtained from each of the five islands surveyed in Hawaii and dot-blot hybridization assays did not pick up all RT-PCR positive samples, the initial study on TaVCV mentioned the virus population in Hawaii maybe highly diverse (Long et al., 2014). However, results from this study point towards very low genetic diversity. There could be a single TaVCV strain which has spread rapidly throughout the state because of the sharing of planting materials

and efficient transmission by a yet to be identified vector. Perhaps, TaVCV may not be as old an introduction into Hawaii as previously thought. Furthermore, the Hawaiian isolates only varied by 1.2 – 2.3% at the nucleotide level and 1 – 2.3% at the protein level versus the Palauan isolates. Similarly, Palauan sequences were nearly identical at both genomic (0 – 0.1% variation) and proteomic levels (0% variation) amongst themselves. Keeping in mind that all samples from Palau were obtained from one location, the country's TaVCV diversity may not be adequately represented.

The Hawaiian and Palauan sequences combined had maximum variability of 22% at the nucleotide level and 8.7% at the protein level against the GenBank Fijian isolate. A previous TaVCV diversity study using the L (RdRp) gene of isolates from Federated States of Micronesia, Fiji, New Caledonia, Papua New Guinea, Solomon Islands and Vanuatu identified maximum variabilities of 27.4% and 11.3% at the nucleotide and protein levels respectively. Individually, Papua New Guinea had the highest within country genomic variation (24.2%); followed by Vanuatu (23.9%), Federated States of Micronesia (16.1%), Solomon Islands (14.3%) and Fiji (3%). Interestingly one of the Fijian isolates, not included in the previous assessment, was 13.9% variable from other isolates in Fiji while diversity values for New Caledonia were not reported (Revill et al., 2005a). This type of high intra-country genetic diversity was not observed in the Hawaiian and Palauan TaVCV isolates.

Then again, there could be a certain primer-induced bias towards the samples that were amplified and identified as positives and later sequenced. Differences in a template's amplification efficiency over another in a mixed template reaction and primer mismatches at their corresponding annealing sites can thereby generate sequence homogeneity (Jabara et al., 2011). Just lately, screening the primer-associated bias of four sets of CTV specific primers,

targeting the A and F regions and the p33 and p23 genes revealed amplification of A and F regions by their respective primer pairs can be highly biased. It was also discovered that p33 gene primers were biased against two genotypes; amplifying them at less than expected levels (Read & Pietersen, 2016). This is not to say the Hawaiian and Palauan TaVCV isolates may have been affected in a similar way, but to put forward a point of consideration in reference to the little diversity noted in the sequence information of the samples studied.

Low nucleotide sequence variabilities were also supported by statistical analyses. Values for nucleotide diversity were extremely minor and statistically insignificant when tested by 3 different algorithms: Jukes-Cantor test, Fu & Li's test and Tajima's test (Table 2.2). Some of these values could not be calculated for Oahu, Molokai and Palau as two or four and more sequences were required for the different algorithms. Despite the shortfall, a clear low diversity status could be observed in all Hawaiian islands, the State itself as well as Palau.

Outside of the *Rhabdovirus* family, analysis of the coat-protein (CP) gene of *Maize dwarf mosaic virus* (MDMV) (genus *Potyvirus*; family *Potyviridae*) isolates from Hungary showed high inter-isolate diversity with MEGA *p*-distances ranging from 0.000 to 0.136 (Gell, Balázs, & Petrik, 2010). On the contrary, isolates of Spanish *Watermelon mosaic virus* (WMV), also a *Potyvirus* were found to be less variable over the P1, CI and CP regions of their genomic RNA with very low nucleotide diversity values: 0.0184, 0.0308 and 0.0232 respectively, although the method or model used to obtain these values were not made clear (Moreno et al., 2004). Similarly, analysis of the CP gene of *Southern rice black-streaked dwarf virus* (SRBSDV) (genus *Fijivirus*; family *Reoviridae*) showed very little diversity; the isolates had >97.8% nucleotide and amino acid homology (Cheng et al., 2013). However, this conclusion was based upon assessment of percent identities alone and not calculations of nucleotide diversity.

Diversity studies on plant infecting nucleorhabdoviruses are rare. None-the-less, two cytorhabdoviruses have been studied in the same regard. In the first case, two distinct clusters or subgroups of LNYV were discovered upon phylogenetic analysis of the complete N gene of eight isolates across Australia. The genomic sequences in each of the subgroups were less than 4% variable but close to 20% different between the two groups. Their translated amino acid sequence variation was less than 4% suggesting a strong preservation force might be acting in favor of the function of this protein (Callaghan & Dietzgen, 2005; Higgins et al., 2016). Similarly, two subgroups of SCV were also discovered via phylogenetic analysis of the L gene of eight isolates from Europe. Much like LNYV, approximately 98% identity existed within nucleotide sequences of the two subgroups, but differed by around 11% between the two subdivisions (Klerks et al., 2004). These patterns were obviously not observed in the Hawaiian and Palauan isolates.

In this current study, all the Hawaiian isolates always grouped tightly together in a super-clade in both nucleotide and deduced amino acid phylogenetic analyses without any hint of forming subgroups. The Palauan isolates segregated into a group of their own while the lone Fijian isolate seemed to form a third group. This style of distribution points towards separation by geographic origin, however, definite implications cannot be made unless more isolates are sequenced from each of the Pacific island countries reported to harbor TaVCV. More support towards this observation could have been obtained if partial L (RdRp) gene sequences from the six PICs used for variability studies by Revill et al. (2005a) were made available. Their analyses did indicate a general tendency of the isolates to group by country but with many exceptions. Indeed, sharing of taro germplasm between Pacific nations does underpin this finding.

To this date, *Citrus tristeza virus* (CTV), a *Closterovirus* , is the only other plant virus to have a genetic variability study conducted in Hawaii (Melzer et al., 2010). Although high genetic diversity was reported in Hawaiian CTV by the authors (π(JC) values of up to 0.0657 for the CP gene and 0.0879 for the p23 gene), the same cannot be said for Hawaiian and Palauan TaVCV. This is the second only study of plant virus genetic diversity in Hawaii and the first for a *Nucleorhabdovirus*. A thorough survey of PICs may reveal more information on the diversity, evolutionary relationships and spread of the disease and even help identify new territories or countries that could now possibly have the virus.

CHAPTER 3: DEVELOPMENT OF IMPROVED DETECTION ASSAYS FOR *TARO VEIN CHLOROSIS VIRUS* (TAVCV)

Introduction

Polymerase chain reaction (PCR) is a widely used molecular assay in which small amounts of DNA or RNA (after reverse transcription into cDNA) can be amplified and utilized for the detection of plant viruses with great specificity and sensitivity (Saiki et al., 1988; Schochetman, Ou, & Jones, 1988; Bej & Mahbubani, 1992). On the other hand, enzyme-linked immunosorbent assay (ELISA) is a serological 'micro-titer plate' based technique used to detect viral proteins (hence the presence or absence of virus in a sample) by trapping them with antibody raised against the virus of interest (Clark & Adams, 1977).

While general principles and functionality of these methods for the detection of plant pathogens remain the same, several variations of PCR and ELISA or the combination of these techniques have been described (Naidu & Hughes, 2003; Webster, Wylie, & Michael, 2004). In recent years, polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) have gradually become the prominent techniques for the detection of taro viruses (Revill et al., 2005b; Babu et al., 2011; Kazmi et al., 2015; Meli & Atibalentja, 2016). ELISA, so far, has only been described for the detection of *Dasheen mosaic virus* (Hu, Meleisea, & Wang, 1994; Hu et al., 1995).

In the initial survey for *Taro vein chlorosis virus* (TaVCV) in Hawaii conducted by Long et al. (2014) using the RT-PCR technique, it was noted that the published TaVCV specific primer set Pol2A1/Pol2A2 (Revill et al., 2005a) detected TaVCV in 8 samples that did not show typical symptoms of the disease. This meant that the detection assay with this particular primer

pair could detect the virus before the infected plants showed symptoms or after recovery from symptoms. More interestingly, Pol2A1/Pol2A2 failed to detect TaVCV in 3 plants that displayed typical disease symptoms. While this points to some diversity within the L (RdRp) gene of TaVCV isolates at the nucleotide level, at least in the conserved regions where the primers anneal to, it may also mean the RT-PCR assay with primer set Pol2A1/Pol2A2 is not very sensitive. In the latter case, if this primer pair is used for future surveys, many samples may produce false negatives while low titer isolates may in fact not be detected at all.

Additionally, previously published primer sets Pol2A1/Pol2A2 and TaVCV1/TaVCV2 (Revill et al., 2005a), both targeting different regions of the L (RdRp) gene, have not been evaluated for sensitivity. Field observations of TaVCV have indicated that symptoms of infected plants may not be displayed continually. In other words, new emerged leaves after the initial series of diseased leaves have senesced may not show TaVCV symptoms at all (Harding, n.d.). The same phenomenon was noted in taro planted in the greenhouse intensifying the need for a more reliable and efficient detection method. For these reasons, development of new TaVCV specific primers and scrutinizing them against each other and existing primer sets became important.

An RT-PCR assay is the only detection method that currently exists for TaVCV. The requirement of time-consuming procedures for sample preparation (total RNA extraction) maybe an obstacle for large scale TaVCV surveys and routine screening in Hawaii; more so in Pacific Island countries which lack specialist laboratories, equipment and expertise. Furthermore, RNA extraction followed by cDNA synthesis before PCR is done may also allow room for errors and contamination; especially when undertaken by less-experienced personnel. To avoid these and for high-throughput processing of samples, a serological technique such as ELISA and a

combined serological-molecular assay such as immunocapture-RT-PCR (IC-RT-PCR) is more suitable. This chapter describes the development of a highly sensitive TaVCV RT-PCR protocol with a new TaVCV specific primer set and efforts made to establish an indirect-ELISA (ID-ELISA) assay for rapid TaVCV detection. Successful establishment of an IC-RT-PCR assay is also discussed.

Materials and Methods

Part I

TaVCV sequences

The partial L (RdRp) gene sequences of 42 Hawaiian TaVCV isolates were obtained from previous work described in CHAPTER 2. The partial L (RdRp) gene sequence of a Molokai isolate was obtained from GenBank (accession KF921086) and sequence information for a fully characterized TaVCV isolate from Fiji was also obtained from GenBank (accession AY674964).

Design of TaVCV specific primers

Primers were designed based on specificity and compatibility with each other.

Approach 1: the online Cap 3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3) was used to create a consensus sequence of the partial L (RdRp) gene sequence from all the 42 Hawaiian isolates. This 'overall consensus' sequence was used to generate four primer sets using three online software programs: Primer-BLAST NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) [two pairs named DCGF1 (F)/ DCGR1 (R) and DCGF2 (F)/ DCGR2 (R)], BiSearch: Primer Design and Search Tool (http://bisearch.enzim.hu/) [one pair named DCGF3

(F)/ DCGR3 (R)], and Primer3plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) [one pair named DCGF4 (F)/ DCGR4 (R)] (Table 3.1).

Approach 2: the 42 partial L (RdRp) gene sequences of Hawaiian TaVCV isolates, the partial L (RdRp) gene sequence of the Molokai isolate and the fully characterized TaVCV genome of a Fijian isolate were aligned using the ClustalX 2.1 software (Larkin et al., 2007). Two primer pairs were manually designed from conserved regions of this multiple sequence alignment and named DCGF5 (F)/ DCGR5 (R) and DCGF6 (F)/ DCGR6 (R) respectively (Table 3.1). All of the above six primer pairs were evaluated using the Multiple Primer Analyzer feature at the ThermoFisher Scientific website for primer-dimer detection and secondary structure formation (www.thermofisher.com). The primers were then ordered from Integrated DNA Technologies® (http://www.idtdna.com/site).

RT-PCR optimization

The six new primer sets were initially tested against an infected sample from Oahu named TO6-8 that was earlier identified as TaVCV positive by the published primer set Pol2A1 (F)/Pol2A2 (R) (Revill et al., 2005a). The PCR protocol employed here was the same as described in CHAPTER 2.

A gradient PCR was then performed at six different annealing temperatures; 48°C, 50°C, 53°C, 55°C, 58°C and 60°C, respectively, to determine the best annealing temperature for each of the 6 primer sets. Additional optimization of primer set DCGF5/DCGR5 included higher annealing temperature gradients; 63°C, 65°C, 68°C and 70°C followed by primer concentration gradients; 10 µM, 5 µM, 2.5 µM and 1 µM per reaction. In addition, the appropriate number of

amplification cycles was evaluated (30, 32 or 35 cycles) using the optimal annealing temperature and primer concentration.

Sensitivity test

To determine the detection limits of the six new primer sets in a standard PCR assay, TaVCV cDNA for the positive control TO6-8 was serially diluted tenfold ($10^0 – 10^{-7}$) and tested with each of the 6 primer pairs. For evaluation, previously published TaVCV specific primer sets Pol2A1/Pol2A2 and TaVCV1 (F)/TaVCV2 (R) (Revill et al., 2005a), both targeting different regions of the L (RdRp) gene were also included in this assessment.

Testing Hawaiian isolates

To compare the RT-PCR assay developed in this study with previously published assay, the cDNA of 208 samples collected from Hawaii Island, Maui and Oahu that had previously been tested with primer pair Pol2A1/Pol2A2 (results reported in CHAPTER 1) (Long et al., 2014), were re-tested with primer set DCGF5/ DCGR5.

Testing Palauan isolates

The eight samples from Palau that were earlier tested with primer set Pol2A1/Pol2A2 and had three out of the eight samples identified as TaVCV positive (sequenced; described in CHAPTER 2) were re-tested with primer set DCGF5/DCGR5 utilizing the optimized protocol described in Part I of the results section.

Part II

TaVCV Antibody Production

The nucleocapsid (N) gene of 18 Hawaiian TaVCV isolates were amplified using primer set Cap2A (F)/Cap2B (R) (Revill et al., 2005a). This primer pair targets a 1.05 kb region of the N gene. All the 18 isolates (9 from Maui, 8 from Kauai and 1 from Oahu) were sequenced as previously described in CHAPTER 2 and their nucleotide sequences were translated into amino acid sequences at the ExPASy website (http://web.expasy.org/translate/). Protein sequence information for the N protein of the Fijian isolate was obtained from GenBank (accession YP_224078). This protein sequenced was analyzed for its antigenic properties alongside the predicted protein sequence of sample TK2-2 from Kauai. Kyte-Doolittle hydropathy plots were constructed using the 'Peptide Finder' application at the Protein Lounge website (http://www.proteinlounge.com/Tool/ Peptide_Finder/Landing.aspx). The peptide sequence of the Fijian isolate (Appendix B) was submitted to GenScript® USA Inc. (Piscataway, NJ) for polyclonal antibody production.

Collection of Samples

The TaVCV positive and healthy plant samples were collected from University of Hawaii's Waimanalo Research Station, Waimanalo, HI in January 2016 (labelled WAK1 – WAK21) and August 2016 (labelled WPT1, WPT2 and WPT-GR). Their TaVCV status was confirmed by RT-PCR using the optimized protocol with primer set DCGF5/DCGR5 as previously described. Whole plant samples collected in January 2016 were planted in an insect-proof greenhouse while two of the three samples gathered in August 2016 were kept in a freezer at -20°C; one sample was planted and kept in an insect cage in a growth room (labelled WPT-

GR). Four taro plants from University of Hawaii's Kona Research Station, Kainaliu, HI (labelled TK1 – TK4) were received in March 2016 and planted. All Waimanalo and all Kona plant samples underwent tissue culture using the protocol described by Keolanui, Sanxter, & Hollyer (1993). Four plants (WAK5, WAK 6, WAK16 and WAK17) from the 21 Waimanalo samples and their tissue cultured propagules were RT-PCR positive for TaVCV while the initial plants obtained from Kona as well as their tissue cultured propagules were RT-PCR negative for TaVCV. Taro leaf samples from Molokai (labelled M1 – M7) were obtained in October 2016. One taro plant (labelled HDT1) was purchased from a nursery in Honolulu, HI. Non-host plant samples were randomly collected from around University of Hawaii – Manoa campus. These were dumb cane (*Dieffenbachia sp.*), hibiscus (*Hibiscus sp.*), papaya (*Carica papaya*), 'ape (*Alocasia macrorrhizos*) and devil's ivy or pothos (*Epipremnum aureum*). Taro planthopper (*Tarophagus spp*., labelled PH-GR) samples were collected from the Waimanalo symptomatic plant kept in the growth room.

ID-ELISA

ID-ELISA was performed using polyclonal TaVCV antisera. First, 0.5 g of healthy or virus infected leaf tissues were homogenized in 5 mL carbonate coating buffer [Na_2CO_3 (anhydrous) 1.59 g, $NaHCO_3$ 2.93 g, NaN_3 0.2 g, ddH_2O to 1 L, pH 9.6]. Then 100 µL of the diluted sample extracts were used to coat the wells of a 96-well microtiter plate and incubated at 37°C for 2 hours (alternatively at 4°C overnight) then washed 3 times with PBST (10X PBS 100 mL, Tween 20 0.5 mL, ddH_2O 900 mL; 10X PBS = NaCl 80.0 g, KCl 2.0 g, $Na_2HPO4.2H_2O$ 14.4 g, KH_2PO4 2.4 g, NaN_3 0.2 g, ddH_2O to 1 L, pH 7.4). Free antigenic peptide artificially synthesized from TK2-2 protein sequence information (GenScript®) was always used as positive

control. The wells were blocked with 200 µL of blocking buffer (100 ml PBST, 1 g bovine serum albumin), incubated at 37°C for 1 hour (alternatively at 4°C overnight) then washed 3 times with PBST. Diluted polyclonal TaVCV antisera (100 µL) was added per well and incubated at 37°C for 2 hours (alternatively at 4°C overnight) then washed 3 times with PBST. Pre-immune serum or ddH_2O were used as antibody negative control, that is, antigen coated wells were loaded with pre-immune serum or ddH_2O instead of polyclonal TaVCV antisera. Next, 100 µL of diluted goat-∞-rabbit IgG conjugate (detection antibody) was added per well, incubated at 37°C for 2 hours then washed 5 times with PBST. Lastly, 100 µL substrate [5 mg phosphatase tablet (Sigma-Aldrich®, St. Louis, MO) dissolved in 5 mL PNP substrate buffer; $MgCl_2.6H_2O$ 0.1 g, NaN_3 0.2 g, Diethanolamine 97 mL, ddH_2O to 1 L, pH 9.8] was added to each well. OD readings were taken at 405 nm after 15, 30, 45 and 60 minutes.

IC-RT-PCR

PCR tubes were coated with 100 µL diluted Anti-TaVCV-N-1 polyclonal antisera (in carbonate coating buffer), incubated at 37°C for 2 – 3 hours then washed 3 times with PBST. Approximately 0.1 g of leaf tissue was homogenized in 1 mL PBST and centrifuged at 4500 *g* for 2 minutes. Then, 100 µL of the diluted supernatant (also in PBST) were dispensed into the PCR tubes, incubated at 4°C overnight then washed 3 times with PBST and 1 time with ddH_2O. The immunocapture was followed by RT and PCR using primer set DCGF5/DCGR5 with the optimized protocol described in Part I the results section.

Results

Part I

New TaVCV specific primers and initial tests

A total of six new TaVCV specific primer sets targeting different regions of the L (RdRp) gene were designed; primer details are provided in Table 3.1 below.

Table 3.1. Primers designed for the specific detection of TaVCV by standard RT-PCR. Sets 1 and 2 were generated by the online software Primer-BLAST NCBI, Set 3 by BiSearch: Primer Design and Search Tool and Set 4 by Primer3plus. Sets 5 and 6 were manually created from ClustalX 2.1 multiple sequence alignment of 44 TaVCV isolates. The start and stop numbers represent nucleotide positions within the 952 bp region of the L (RdRp) gene of TaVCV sequenced as part of this study (see Appendix A).

Y = C/T; R = A/G; S = G/C; M = A/C; H = A/C/T; W = A/T; K = G/T

Set #	Primer Name	Sequence 5' – 3'	Start	Stop	Product Length (bp)	T_m (°C)	GC Cont. (%)
1	**DCGF1**	CCTGACGGAGTATGGTCGTG	126	145	544	59.90	60
	DCGR1	ACAACTCTGCCACCCTTCTG	669	650		59.89	55
2	**DCGF2**	GTGGACCGGTGATGAGTCTG	143	162	368	60.11	60
	DCGR2	TTGCTTTCAAGGGGGACCTC	510	491		59.89	55
3	**DCGF3**	GTCTGGTAAAGAGGGGTTGA	157	176	399	61.1	50
	DCGR3	GTCGAGGGTCATGATAGAGT	556	537		60.6	50
4	**DCGF4**	ATCCAGGATATTCCCCTTGG	512	531	240	60.0	50
	DCGR4	CTCTTTCCCGCTCTTGTCAC	751	732		60.0	55
5	**DCGF5**	AGGGGYTGAGRCAAAAGGGGT	168	188	442	64.5	59
	DCGR5	CGCTCYTTCATACATGCSGCCTT	609	587		63.0	54
6	**DCGF6**	TGATTGCMAAGMGACATCATGTHG	219	242	509	57.6	43
	DCGR6	ATGATCWGGCARCTCKGGCTT	727	705		60.6	52

When initially tested with the PCR protocol outlined in CHAPTER 2, all the six new primer sets efficiently detected TaVCV from the positive control sample TO6-8. This sample was originally identified as positive by the Pol2A1/Pol2A2 primer set (Fig. 3.1).

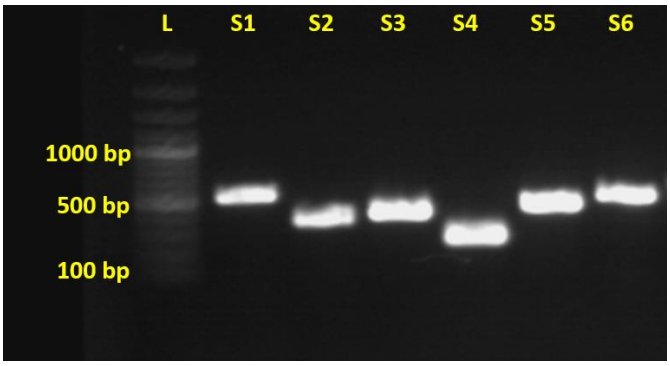


Fig. 3.1. The six new primer sets effectively detected TaVCV from the positive control sample TO6-8 collected from the island of Oahu. S1 = DCGF1/DCGF2, S2 = DCGF2/DCGR2, S3 = DCGF3/DCGR3, S4 = DCGF4/DCGR4, S5 = DCGF5/DCGR5, S6 = DCGF6/DCGR6. L = Ladder lane.

Primer set optimization

With the use of gradient PCR, it was identified that an annealing temperature of 60°C was adequate for all the six new primer sets (Fig. 3.2a – f). Annealing temperatures above 60°C were not trialed at this stage to keep within the ≤ 5°C range of the melting temperatures of individual primers as shown in Table 3.1.

After this primary optimization step, the cycling parameters for TaVCV RT-PCR with the new primer sets were determined to be as follows: initial denaturing at 95°C for 5 minutes and then 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, with a final extension of 72°C for 5 minutes.

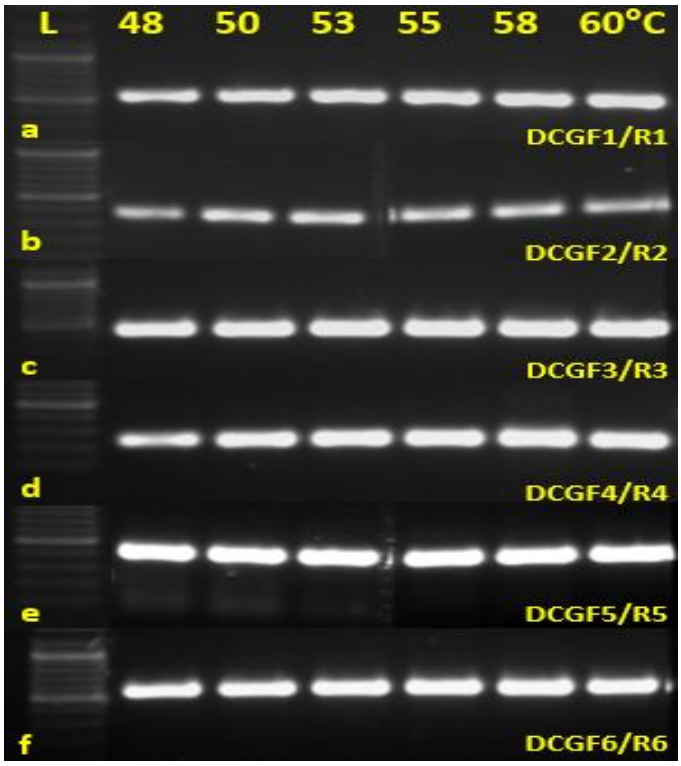


Fig 3.2a – f. Gradient PCRs to confirm the optimal annealing temperature for each of the six new primer sets. All the primer pairs amplified TaVCV efficiently up to 60°C annealing temperature. L = Ladder lane.

Tests on Molokai isolates

The 23 Molokai isolates identified as TaVCV positive by Long et al. (2014) were of particular interest because they produced extremely weak TaVCV positive bands when PCR was re-conducted with primer set Pol2A1/Pol2A2. As a result of this low amplification, none of the Molokai samples could be sequenced even though agarose gel fragments were excised, eluted and then used in downstream applications.

From these 23 isolates, six samples were randomly tested with all the six new primer sets employing the PCR protocol described above. All of the six new primer sets detected TaVCV from Molokai samples TMol1-1, TMol1-6, TMol1-18, TMol1-20, TMol2-9 and TMol2-13 (Fig.

3.3a – f respectively) except set 6 from sample TMol1-6. The Pol2A1/Pol2A2 primer set produced an amplification product (952 bp) in only three of the six samples. For comparison, PCR with primer set Cap2A/Cap2B was also conducted. This primer pair detected TaVCV in two out of the six positive Molokai samples (Fig. 3.3a – f).

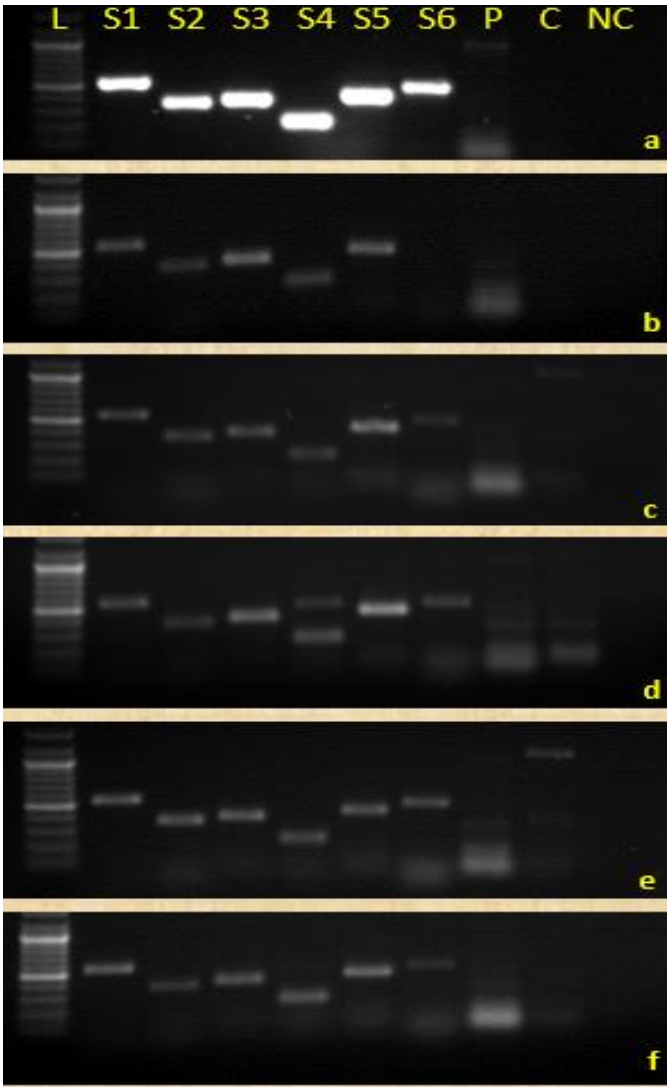


Fig: 3.3a – f. The six new primer sets effectively detected TaVCV from six randomly chosen (a – f) Molokai samples. S1 = DCGF1/DCGF2, S2 = DCGF2/DCGR2, S3 = DCGF3/DCGR3, S4 = DCGF4/DCGR4, S5 = DCGF5/DCGR5, S6 = DCGF6/DCGR6, P = Pol2A1/Pol2A2, C = Cap2A/Cap2B, L = Ladder lane, NC = Negative Control.

Sensitivity of the TaVCV RT-PCR assay

To compare the relative sensitivity of the six newly designed as well as the Pol2A1/Pol2A2 and TaVCV1/TaVCV2 primer sets, a series of tenfold dilutions of positive control template for TaVCV (cDNA; TO6-8) was analyzed by RT-PCR. The highest TaVCV sensitivity was noted for primer set DCGF5/DCGR5; the virus-specific band was observed after the positive control template was diluted to an endpoint of 10^{-5}. Primer set TaVCV1/TaVCV2 failed to detect TaVCV at all while Pol2A1/Pol2A2 only managed to amplify the positive control template diluted to an endpoint of 10^{-1} (Fig 3.4). Because primer set DCGF5/DCGR5 outperformed all other primer pairs, its PCR product was treated with ExoSAP-IT® PCR Product Cleanup reagent (Affymetrix, Santa Clara, CA) and sequenced at the Advanced Studies in Genomics, Proteomics and Bioinformatics Laboratory. Closest identities were noted with the Molokai and Fijian TaVCV isolates followed by other *Nucleorhabdoviruses* upon blastn analysis at the NCBI website. This confirmed the DCGF5/DCGR5 amplicons were indeed TaVCV specific.

Test on Hawaiian isolates

A total of 208 samples from Hawaii Island, Maui, and Oahu were re-tested with primer set DCGF5/DCGR5. There was a 16.4% increase in the number of positive samples from Hawaii Island and a 59.1% increase in the Maui samples. The number of positive samples detected by the two primer sets from Oahu remained the same. Overall, a 26.4% increase in virus incidence was noted in the three islands (Table 3.2).

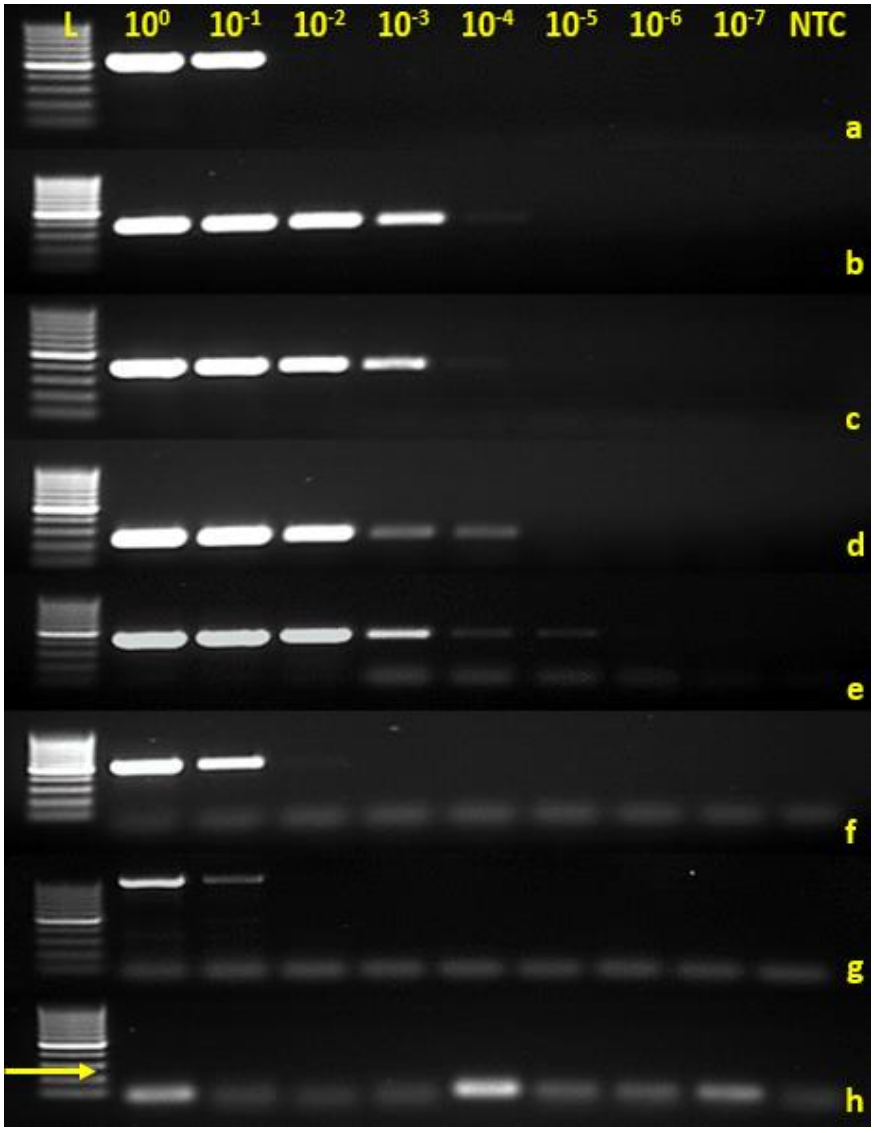


Fig. 3.4a – h: Sensitivity PCRs to determine the detection limits of each of the six new and two previously published primer sets. Pol2A1/Pol2A2 at best amplified the positive control template diluted to 10^{-1} (g), DCGF1/DCGR1 up to 10^{-1} (a); DCGF2/DCGR2 to 10^{-4} (b), DCGF3/DCGR3 up to 10^{-4} (c); DCGF4/DCGR4 to 10^{-4} (d), DCGF5/DCGR5 to 10^{-5} (e); DCGF6/DCGR6 to 10^{-2} (f) while TaVCV1/ TaVCV2 did not detect TaVCV in the positive Hawaiian sample TO6-8 (h). The yellow arrow represents the size of the expected amplification product (220 bp) of primer pair TaVCV1/TaVCV2.

Table 3.2. Comparison of RT-PCR test for TaVCV in samples collected from three Hawaiian islands using primer set Pol2A1/Pol2A2 and DCGF5/DCGR5. The value in brackets indicate the percentage of positives in relation to the total number of samples tested.

Primer / Island	Big Island	Maui	Oahu	Overall
Pol2A1/Pol2A2 (initial survey)	7/79 (8.9%)	19/71 (26.8%)	2/58 (3.4%)	28/208 (13.5%)
DCGF5/DCGR5	20/79 (25.3%)	61/71 (85.9%)	2/58 (3.4%)	83/208 (39.9%)

Additional optimization of primer set DCGF5/DCGR5

Testing field isolates and some tissue cultured taro samples with primer set DCGF5/DCGR5 using the PCR protocol described above showed it may sometimes produce a non-specific band. In these samples, a second shorter fragment ~250 bp, in addition to the 442 bp DCGF5/DCGR5 product would get amplified (Fig. 3.5a). Since DCGF5/DCGR5 was identified as the most sensitive and robust of the eight TaVCV specific primer sets, this pair was further optimized. New annealing temperature gradients revealed 65°C and 68°C were both allowing the primers to bind and amplify the target with comparable efficiency (Fig. 3.5b). Testing these two annealing temperatures with four different primer concentrations; 10 µM, 5 µM, 2.5 µM and 1 µM per reaction, helped determine 5 µM primer concentration per reaction is optimal for both 65°C and 68°C annealing temperatures (Fig. 3.5c, d respectively). These two combinations (5 µM primer concentration/ 65°C annealing temperature and 5 µM primer concentration/68°C annealing temperature) were then trialed at 30, 32 and 35 cycles. Just by band intensity alone, amplification seemed consistent through the 30, 32 and 35 cycles for each of the above two respective combinations (Fig. 3.5e).

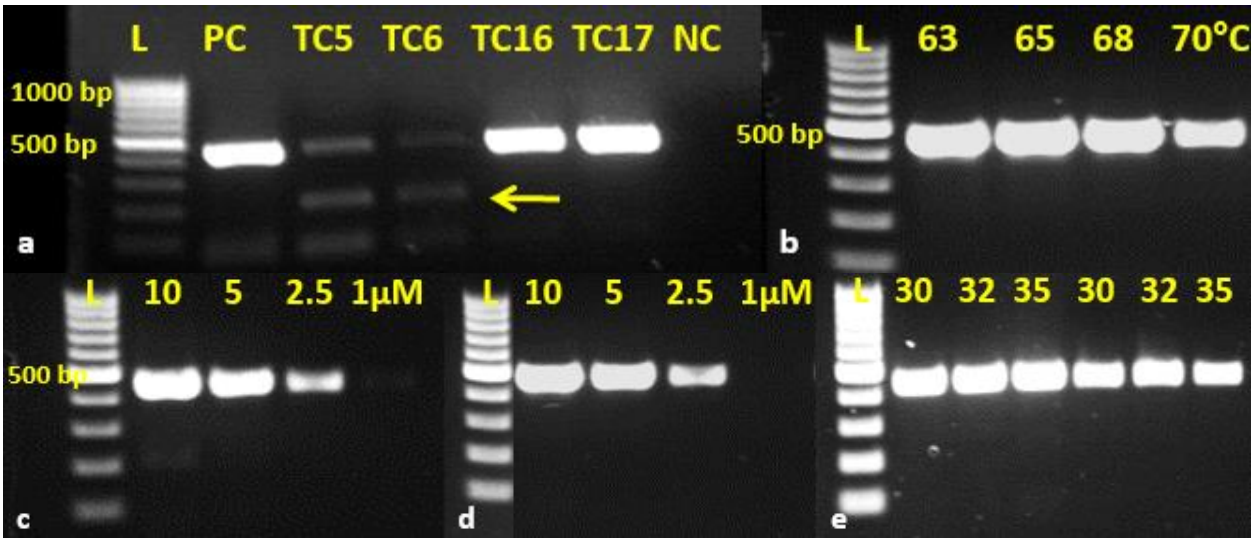


Fig. 3.5a – e. Primer set DCGF5/DCGR5 sometimes produced a non-specific band of ~250 bp (a). New temperature (b), primer (c, d) and cycle gradient (e) PCRs were set up for this primer pair. An annealing temperature of 68°C and primer concentration of 5 µM per reaction were identified as appropriate in a 35 cycle PCR for this primer pair.

The optimized PCR reaction components are provided in Table 3.3 and the cycling conditions are provided in Fig. 3.6.

Table 3.3. Optimized PCR reaction system

Component	**Volume (µL)**
Template (cDNA)	1
DCGF5 (5 µM)	1
DCGR5 (5 µM)	1
2x GoTaq Green Master Mix	10
ddH_2O	7
Total	**20**

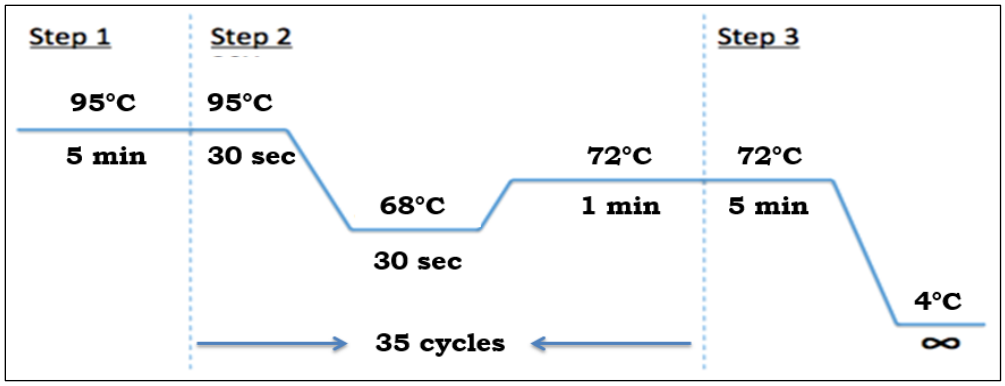


Fig. 3.6. The standard PCR protocol determined for primer set DCGF5/DCGR5 after appropriate optimization steps.

Tests on Palau isolates

Testing the 8 Palauan samples with primer set DCGF5/DCGR5 using the above-mentioned optimized PCR protocol yielded 5 TaVCV positive samples (Fig. 3.7b). This was two more than the number of TaVCV positive samples detected by the published TaVCV specific primer set Pol2A1/Pol2A2 (Fig. 3.7a).

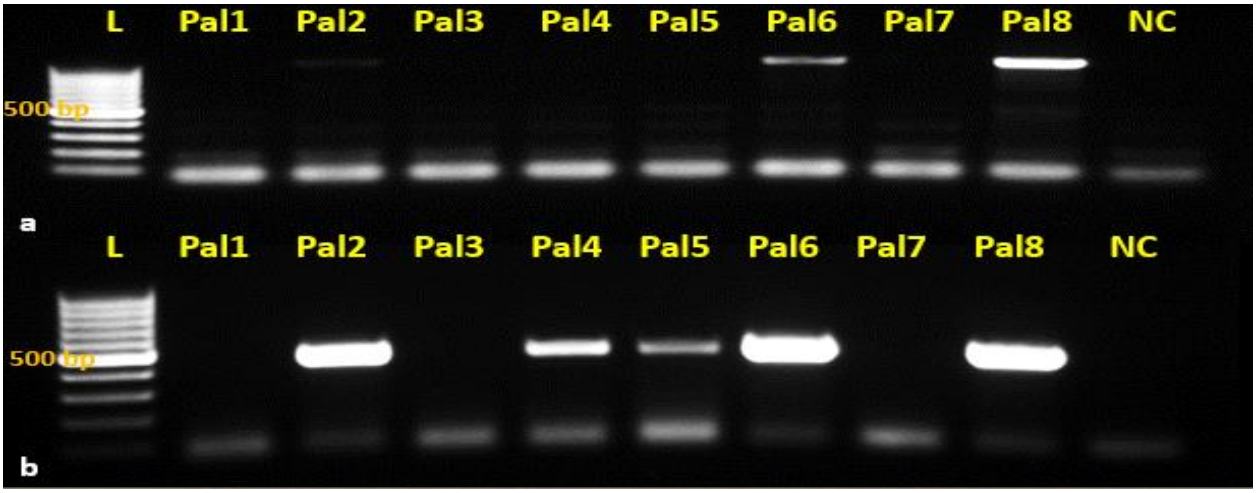


Fig. 3.7a – b. PCR on 8 Palauan taro samples. Primer set Pol2A1/Pol2A2 detected TaVCV in only 3 samples; Pal2, Pal6 and Pal8 (a). Primer set DCGF5/DCGR5 identified two additional positives; Pal4 and Pal5 (b).

Part II

TaVCV Antisera

Constructing Kyte-Doolittle hydropathy plots of the N protein of the Fijian isolate from GenBank (accession YP_224078) and partial predicted amino acid sequence of one of the Kauai isolates (TK2-2) helped confirm the presence of hydrophilic regions (Fig. 3.8). Theoretically, these regions (or high negative x-axis values) indicate the hydrophilic residues are in contact with solvent or water, therefore, they are possibly located on the exterior surface of the protein (Kyte & Doolittle, 1982) and can behave as target epitopes in ELISA assays. Three affinity-purified polyclonal antibodies and the respective antigens used to raise these antibodies were received in June 2016, the details of which are provided in Table 3.4.

Table 3.4. Anti-TaVCV polyclonal antibodies produced in New Zealand rabbits by GenScript USA Inc. The antigens (free peptides) were received in lyophilized form and reconstituted in PBS, pH 7.4. The antibodies were received in liquid form, already reconstituted in PBS, pH 7.4 with 0.02% Sodium Azide. They were aliquoted in 5 and 10 µL quantities and stored at -20°C until use.

Antigen Name	Antigen Sequence	Antibody Name	Antibody Conc. & Quantity
TaVCV-N-1	CQQAEGHPTKKRTWK	Anti-TaVCV-N-1	0.855 mg/mL, 3.42 mg
TaVCV-N-2	CRHVPQGVGDYADPR	Anti-TaVCV-N-2	1.258 mg/mL, 5.03 mg
TaVCV-N-3	CTLQLEDQQQRRLSP	Anti-TaVCV-N-3	0.508 mg/mL, 2.03 mg

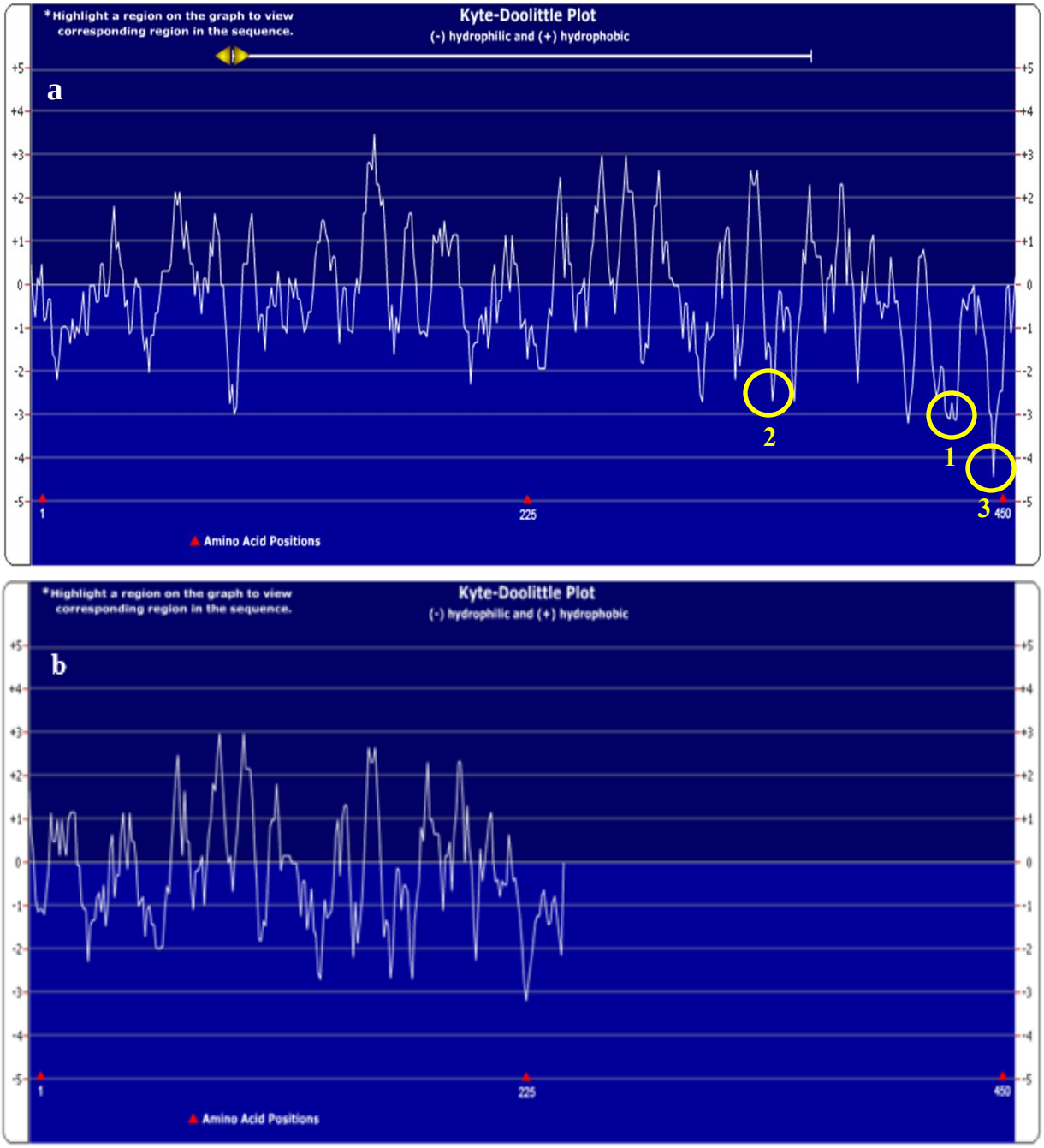


Fig 3.8a – b. Kyte-Doolittle plots to analyze the areas of hydrophilicity (-) and hydrophobicity (+) for the N protein sequence of a Fijian isolate (a) and partial N protein sequence of sample TK2-2 from Kauai (b). Both graphs show regions of high hydrophilicity within the peptide sequences. The circles in Fig. 3.8a represent antigenic regions used to raise the respective antisera mentioned in Table 3.4.

ID-ELISA

Initial ID-ELISA using asymptomatic and symptomatic TaVCV leaf samples with all the three polyclonal antibodies indicated the potential usefulness of Anti-TaVCV-N-1 antisera while Anti-TaVCV-N-2 and Anti-TaVCV-N-3 antisera showed no reactivity, that is, no binding activity towards samples containing diluted extracts from symptomatic leaf tissues (data not shown). Anti-TaVCV-N-2 and Anti-TaVCV-N-3 plates showed no color development while OD_{405} values were extremely low. There was no clear distinction between wells containing asymptomatic and symptomatic TaVCV leaf samples (data not shown).

Several combinations of leaf extract, Anti-TaVCV-N-1 polyclonal antisera and detection antibody (enzyme-linked conjugate) dilution were trialed. Dilution rates of 1:20 for leaf extracts (in carbonate coating buffer), 1:1000 for Anti-TaVCV-N-1 polyclonal antisera (in blocking buffer) and 1:2000 for goat-∞-rabbit IgG conjugate (also in blocking buffer) were identified as optimal. Using this combination, TaVCV was effectively detected from a symptomatic and RT-PCR positive plant from Waimanalo and clearly distinguished from a non-host plant, dumb cane (Table 3.5). However, a major concern was pre-immune serum reactivity with infected plant extract. Normally, pre-immune serum would be expected to be free of any antibodies against TaVCV. Consequently, the wells where pre-immune serum was added instead of Anti-TaVCV-N-1 polyclonal antisera were expected not to turn out positive or develop color. Yet, very high OD_{405} readings were recorded for pre-immune serum wells and color development was also intense, at a rate similar to positive control wells (Table 3.5). For this reason, ddH_2O was used as negative control for the antisera in subsequent tests.

Expanding the evaluation to include more taro and non-host samples showed Anti-TaVCV-N-1 polyclonal antisera produced high OD_{405} values for two RT-PCR tested TaVCV

negative taro samples and two non-host plants, hibiscus and papaya (Table 3.6). Although Anti-TaVCV-N-1 polyclonal antisera seemed promising for establishing an ID-ELISA assay for TaVCV, reactivity with RT-PCR negative samples and non-host plant extracts halted efforts.

IC-RT-PCR

Just like ID-ELISA, different combinations of Anti-TaVCV-N-1 polyclonal antisera dilution and leaf extract dilution were trialed for their IC-RT-PCR suitability. Dilution rates of 1:500 for Anti-TaVCV-N-1 polyclonal antisera (in carbonate coating buffer) and 1:100 for leaf extracts (in PBST) were identified as ideal. Initial tests showed IC-RT-PCR could clearly distinguish between symptomatic TaVCV samples and non-host plants. Using leaf tissue extracts, two symptomatic plants obtained from Waimanalo (WPT1 and WPT2) and one asymptomatic plant purchased commercially (HDT1) were identified as TaVCV positive while non-host plants dumb cane (Dif1), hibiscus (Hib1) and papaya (Pap1) were all TaVCV negative (Fig. 3.9). When the assay was expanded to include more taro and non-host samples, the results were duly reproduced (Table 3.7). However, the highly symptomatic plant from Waimanalo (WPT-GR) kept in the growth room as source plant for rearing TaVCV infected planthoppers was not identified as TaVCV positive. This plant was maintained for 7 weeks before the test with IC-RT-PCR. Interestingly, the same result was obtained by RT-PCR, confirming the IC-RT-PCR result (Table 3.7).

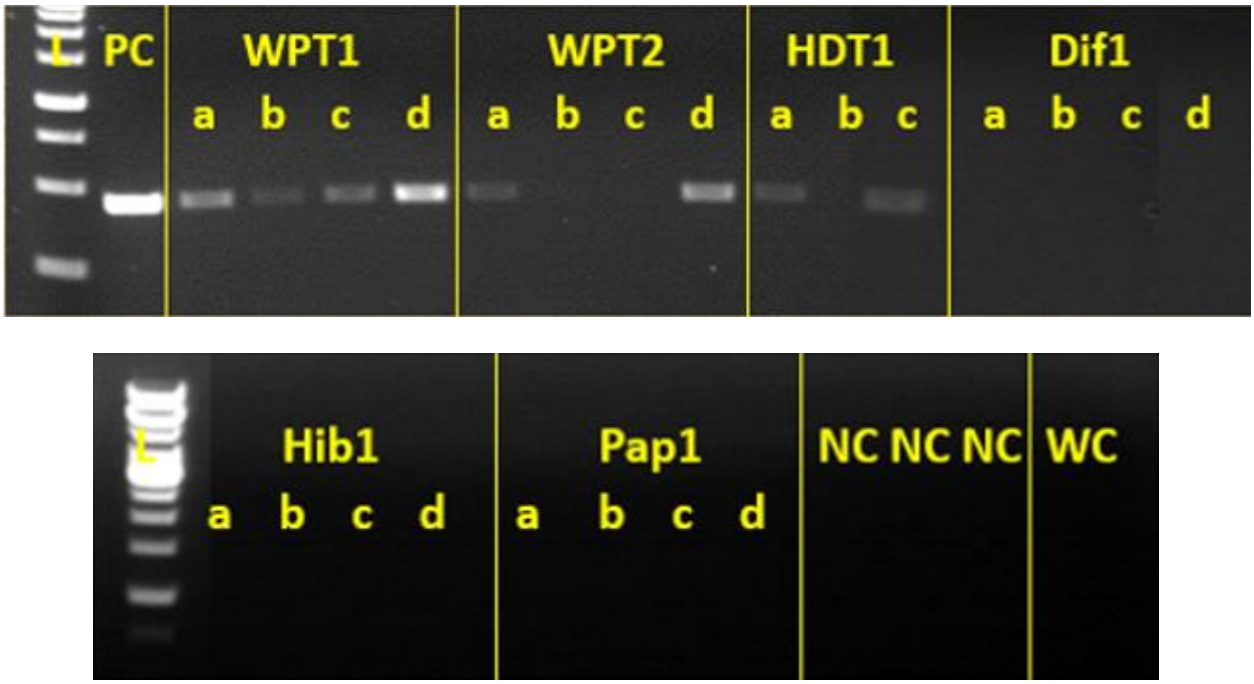


Fig 3.9. IC-RT-PCR analysis of taro and non-host plants. L = ladder lane, PC = positive control, WPT1 & 2a – d = symptomatic taro from Waimanalo, HDT1a – c = asymptomatic commercially purchased taro, Dif1a – d = dumb cane, Hib1a – d = hibiscus, Pap1a – d = papaya, NC = negative control (PCR tubes incubated with ddH_2O instead of Anti-TaVCV-N-1 polyclonal antisera), WC = PCR water control. For every sample, wells a – c were incubated with diluted leaf extract while well d was incubated with crude leaf extract; except for HDTc and the third negative control which were incubated with crude leaf extract.

Table 3.5. ID-ELISA trial with symptomatic, RT-PCR positive sample from Waimanalo (columns 3 – 8) and non-host plant, dumb cane (columns 9 – 11). Leaf extracts diluted at a rate of 1:20, polyclonal antisera at 1:1000 and conjugate at 1:2000 were appropriate (wells 3B, 3C and 6B, 6C). Wells 2F and 2G were incubated with pre-immune serum instead of Anti-TaVCV-N-1 polyclonal antisera, yet these wells surprisingly produced high OD_{405} readings and color development equal to positive control wells 2D and 2E. Dumb cane OD_{405} values were similar to buffer wells.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.135	0.111	0.304	0.131	0.129	0.147	0.183	0.127	0.125	0.142	0.165	0.141	405
B	0.129	0.128	1.763	0.85	0.707	1.493	0.843	0.697	0.346	0.199	0.213	0.15	405
C	0.132	0.13	1.491	0.811	0.694	1.49	0.909	0.86	0.484	0.296	0.371	0.197	405
D	0.157	3.737	0.899	1.512	0.459	0.867	1.572	0.433	0.263	0.391	0.242	0.243	405
E	0.171	3.688	0.915	1.543	0.489	0.91	1.406	0.461	0.252	0.324	0.201	0.213	405
F	0.289	3.644	0.8	0.544	0.19	0.764	0.541	0.259	0.333	0.3	0.256	0.257	405
G	0.271	3.647	0.852	0.56	0.251	0.806	0.532	0.275	0.379	0.308	0.273	0.299	405
H	0.465	0.461	0.449	0.48	0.43	0.437	0.315	0.295	0.321	0.315	0.303	0.298	405

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB
B	CB	CB	A = 1:20 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:2000 DA = 1:2000	A = 1:20 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:2000 DA = 1:2000	A = 1:20 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:2000 DA = 1:2000	CB
C	CB	CB										CB
D	CB	PC	A = 1:20 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:2000 DA = 1:4000	A = 1:20 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:2000 DA = 1:4000	A = 1:20 PA = 1:1000 DA = 1:4000	A = 1:40 PA = 1:1000 DA = 1:2000	A = 1:40 PA = 1:2000 DA = 1:4000	CB
E	CB	PC										CB
F	CB	ANC	A = 1:20 PA = 1:2000 DA = 1:2000	A = 1:20 PA = 1:2000 DA = 1:4000	CB	A = 1:20 PA = 1:2000 DA = 1:2000	A = 1:20 PA = 1:2000 DA = 1:4000	CB	A = 1:20 PA = 1:2000 DA = 1:2000	A = 1:20 PA = 1:2000 DA = 1:4000	CB	CB
G	CB	ANC			CB			CB			CB	CB
H	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB

RT-PCR tested TaVCV +ve plant (Waimanalo)	RT-PCR tested TaVCV +ve plant (Waimanalo) - Replicate	Dumb cane plant from UH-Manoa

Legend:

CB: Coating Buffer Well
PC: Positive Control (Free peptide)
ANC: Negative Control for Antibody (Pre-Immune Serum)
A: Antigen/leaf extract
PA: Primary Antibody
DA: Detection Antibody

Antigen (leaf tissue extraction) diluted at 2 rates = 1:20 and 1:40
Primary antibody tested at 2 rates = 1:1000 and 1:2000
Detection antibody (goat anti rabbit) tested at 2 rates = 1:2000 and 1:4000

Table 3.6. ID-ELISA trial with symptomatic, RT-PCR positive sample from Waimanalo (columns 3 – 4), asymptomatic, RT-PCR positive plant from Waimanalo (column 5), asymptomatic commercially purchased plant (column 6), asymptomatic, RT-PCR negative plants from Kona (columns 7 – 8) and non-host plants dumb cane, hibiscus and papaya (columns 9 – 11). RT-PCR negative plants from Kona and non-host plants hibiscus and papaya produced high OD_{405} readings.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.124	0.109	0.103	0.122	0.099	0.099	0.124	0.225	0.101	0.098	0.098	0.152	405
B	0.128	0.102	1.487	1.466	2.19	0.612	1.883	1.836	0.37	1.16	2.006	0.569	405
C	0.111	0.097	1.479	1.303	2.179	0.623	1.836	1.812	0.383	1.137	2.001	0.233	405
D	0.115	3.674	1.617	1.299	2.058	0.555	1.702	1.866	0.446	1.174	2.124	0.247	405
E	0.136	3.669	1.448	1.386	2.09	0.647	1.748	1.822	0.401	1.314	2.022	0.143	405
F	0.113	0.087	1.559	1.5	2.121	0.642	1.965	1.982	0.416	1.185	2.263	0.395	405
G	0.124	0.086	1.593	1.57	2.353	0.701	1.921	1.963	0.402	1.248	2.203	0.155	405
H	0.142	0.111	0.172	0.137	0.105	0.107	0.106	0.107	0.242	0.119	0.1	0.132	405

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB
B	CB	CB	Waimanalo Positive Leaf 1. A = 1:20, PA = 1:1000, DA = 1:2000	Waimanalo Positive Leaf 2. A = 1:20, PA = 1:1000, DA = 1:2000	Waimanalo Weak Positive. A = 1:20, PA = 1:1000, DA = 1:2000	Home Depot Plant. A = 1:20, PA = 1:1000, DA = 1:2000	Kona Plant 1 Leaf. A = 1:20, PA = 1:1000, DA = 1:2000	Kona Plant 3 Leaf. A = 1:20, PA = 1:1000, DA = 1:2000	Dumb cane Leaf. A = 1:20, PA = 1:1000, DA = 1:2000	Hibiscus Leaf. A = 1:20, PA = 1:1000, DA = 1:2000	Papaya Leaf. A = 1:20, PA = 1:1000, DA = 1:2000	CB
C	CB	CB										CB
D	CB	PC										CB
E	CB	PC										CB
F	CB	ANC										CB
G	CB	ANC										CB
H	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB	CB

Refrigerated samples (-20°C). RT-PCR tested, Strong Positive	RT-PCR Tested, Weak Positive	RT-PCR Tested, TaVCV Negative	Non-hosts, not tested by PCR

Legend:

CB: Coating Buffer Well
PC: Positive Control (Free peptide)
ANC: Negative Control for Antibody (ddH_2O)
A: Antigen/leaf extract
PA: Primary Antibody
DA: Detection Antibody

Antigen/leaf extract = 1:20
Primary antibody = 1:1000
Detection antibody (goat anti rabbit) = 1:2000

Table 3.7. IC-RT-PCR analysis of additional taro and non-host plants. The RT-PCR and IC-RT-PCR were always in agreement except for the taro planthopper samples collected from WPT-GR. WT1a – c = symptomatic taro plant from Waimanalo, HDT1a – c = commercially purchased asymptomatic taro plant, WAK8 and WAK9 = asymptomatic taro plants from Waimanalo, M1 – M7 = asymptomatic taro leaf samples from Molokai, WPT-GR = symptomatic taro plant from Waimanalo kept in a growth room, PH-GR = planthoppers feeding on the symptomatic taro plant kept in the growth room. Y = yes, N = no, NT = not tested, F = faint band in agarose gel electrophoresis, NA = not applicable.

Samples	**Symptoms**	**RT-PCR**	**IC-RT-PCR**
WPT1a – c	Y	+	+
HDT1a – c	N	+	+
WAK8	N	-	-
WAK9	N	-	-
M1	N	-	-
M2	N	-	-
M3	N	NT	-
M4	N	NT	-
M5	N	NT	-
M6	N	NT	-
M7	N	NT	-
‘Ape (non-host)	N	-	-
Devil’s Ivy (non-host)	N	-	-
WPT-GR	Y	-	-
PH-GR	NA	+ (F)	-

Discussion

The accurate diagnosis of a plant virus disease and delineating its geographical as well as temporal distribution in a particular area or crop is a crucial first-step in establishing management and control strategies. This is complicated by the fact that increased international agricultural trade promotes constant movement of germplasm via seed and other forms of propagative material between countries. Diagnosis of virus infections in these resources is vital for quarantine purposes to make sure the exchange of materials occur safely and pose zero to minimal risk of diseases (Naidu & Hughes, 2003). However, such exercises are only possible with the availability of specific and highly robust detection assays that allow screening with maximum efficiency.

The observation by Long et al. (2014) that the published TaVCV specific primer set Pol2A1/ Pol2A2 did not detect TaVCV in 3 symptomatic plants provided initial evidence of the inadequacy of this primer pair for RT-PCR detection of TaVCV in Hawaii, and possibly other locations as well. The need for an improved molecular assay was reinforced when both primer sets Pol2A1/Pol2A2 and Cap2A/Cap2B showed discrepancies in detecting TaVCV in re-tests of Molokai samples that were originally identified as TaVCV positive with Pol2A1/Pol2A2 itself (Fig. 3.3). These cases combined raised serious concerns of obtaining a large number of false negatives if primer sets Pol2A1/Pol2A2 and Cap2A/Cap2B were utilized for monitoring and field surveillance of TaVCV in Hawaii. More so, if used for testing germplasm for international transfer, these would increase the risk of unintended TaVCV spread.

The six new primer pairs designed as part of this study add to the limited resources currently available for TaVCV detection. Primer set DCGF5/DCGR5 was by far the most sensitive of all TaVCV specific primer pairs (Fig. 3.4). It is also more efficient than previously

available primers as demonstrated by its detection capability in an RT-PCR assay of Palauan samples (Fig 3.7). This same primer pair detected 55 more positive samples from a total of 208 samples obtained from Big Island, Maui and Oahu compared to primer set Pol2A1/Pol2A2 reflecting a 26.4% increase in disease incidence (Table 3.2). DCGF5/DCGR5 is recommended for robust molecular detection of TaVCV in future studies, routine monitoring and for inspection needs. It is important to note that RNA extraction and the RT step (or cDNA synthesis) before actual PCR make this assay laborious and time-consuming especially in large scale surveys.

Therefore, a serological technique such as ELISA is preferred for the simple fact that it allows for high-throughput processing of samples. One has to remember, however, the many steps involved, preparation and storage of reagents, incubation time and temperature, sampling of appropriate plant parts and use of suitable extraction, coating and blocking buffers are key elements that affect sensitivity and reliability of this assay (Mclaughlin et al., 1981; Hewings & D'Arcy, 1984); the quality of antisera being the most significant (Clark, 1981). Three polyclonal antisera were raised against TaVCV (Table 3.4) using very short artificially created peptides (15 amino acids) representing different hydrophilic regions of the N protein; coupled with the carrier protein keyhole limpet hemocyanin (KLH); together forming the antigen. This was an alternate approach from the usual polyclonal antibody production techniques where much longer or entire proteins or purified virus extracts are used (Drenckhahn, Jöns, & Schmitz, 1993; Cooper & Paterson, 2009). While Anti-TaVCV-N-2 and Anti-TaVCV-N-3 polyclonal antisera showed no reactivity to TaVCV infected leaf extracts or healthy controls in an ID-ELISA assay, Anti-TaVCV-N-1 did react to TaVCV. The epitopes targeted by Anti-TaVCV-N-2 and Anti-TaVCV-N-3 may in fact not be exposed. On the other hand, Anti-TaVCV-N-1 antisera also reacted to

non-host plants papaya and hibiscus as well as RT-PCR TaVCV negative taro (TK1 and TK3) in follow-up trials (Table 3.6).

A similar antibody production strategy was employed by Garcia, Sanchez, & Montoya (2013) who used a capsid-specific artificial peptide antigen for polyclonal antisera against *Potato mop-top virus* (PMTV; genus *Pomovirus*; family *Virgaviridae*). An ID-ELISA was successfully trialed but negative OD_{405} values recorded for some samples was a concern. Additionally, closely-related and non-host plants were not assayed for cross-reaction. Although pre-immune serum was received by the group, extraction buffer was used as negative control. This suggests Garcia and colleagues may have faced a similar issue with the use of pre-immune serum as antibody negative control much like what was observed in this study. In another research, OD_{405} readings were found not to be variable between infected and healthy samples of *Apple stem pitting virus* (ASPV; family *Betaflexiviridae*) by ID-ELISA when cloned and purified coat protein was used as antigen to make polyclonal antibodies (Jelkmann & Keim-Konrad, 1997). The results obtained by Jelkmann and Keim-Konrad can be correlated with RT-PCR TaVCV positive and RT-PCR TaVCV negative plants showing up as ID-ELISA positive in this study.

Polyclonal antibodies are a collective group of diverse immunoglobulin molecules secreted by a large number of B lymphocyte cells that together react against a specific antigen by targeting different epitopes of the protein with varying affinities (Lipman et al., 2005). If purified virus extracts are used for polyclonal antibody production, the collection may include antibodies against contaminating host-plant material; the source for non-specific binding and ambiguous OD readings in ELISA assays (Naidu & Hughes, 2003). TaVCV purification from taro is relatively difficult (Borth & Hu, personal communication) and for this reason small artificially made peptides from the parent N gene were used. Therefore, in theory Anti-TaVCV-N-1

polyclonal antisera should not react to host (plant) proteins. Its reaction to both RT-PCR TaVCV negative taro and non-host plants suggest other factors are at play.

Cloned viral coat proteins have successfully been used to produce antibodies and develop various serology-based assays for many plant viruses (Folwarczna et al., 2008; Agarwal, Krishna Reddy, & Jain, 2009; Gulati-Sakhujaa et al., 2009; Sivaprasad et al., 2015). Cloned TaVCV nucleocapsid and even glycoprotein can be trialed in future studies. DNA-based immunization methods to raise antibodies against *Tobacco mosaic virus* (TMV; genus *Tobamovirus*, family *Virgaviridae*) coat protein and Potato virus Y (PVY: genus *Potyvirus*, family *Potyviridae*) P1 protein have also shown promise (Hinrichs, Berger, & Shaw, 1997) but did not gain momentum against conventional techniques. The former is recommended over the latter.

Although an ID-ELISA assay for TaVCV could not be resolutely created, Anti-TaVCV-N-1 polyclonal antisera was alternatively used to establish an IC-RT-PCR assay. This technique combines principles from both the serological and molecular domains and proved effective for TaVCV detection. IC-RT-PCR results of taro as well as non-host samples were always in agreement with RT-PCR analysis (Table 3.7). Replicating samples in the IC-RT-PCR assay, that is, testing more than 1 sample per isolate (preferably composites) is suggested. One of the major advantages of IC-RT-PCR is that the assay is less labor-intensive and less time consuming; the long RNA extraction procedure is replaced by a simple tissue homogenization step. The switch from currently used two-step to one-step IC-RT-PCR will make the assay more efficient.

IC-RT-PCR is so far only available for one other plant infecting *Nucleorhabdovirus*, *Eggplant mottled dwarf virus* (Katis et al., 2011). Availability of the improved and highly robust RT-PCR as well as the IC-RT-PCR diagnostic methods established as part of work for this chapter provide greater specificity, increased sensitivity and flexibility for rapid diagnosis of

TaVCV in disease surveys not just in Hawaii but other Pacific countries as well. These assays are good tools for plant quarantine and germplasm distribution centers throughout the taro growing tropics.

CHAPTER 4: EVIDENCE TOWARDS THE TARO PLANTHOPPER [*TAROPHAGUS PROSERPINA* (KIRKALDY)] AS THE VECTOR FOR *TARO VEIN CHLOROSIS VIRUS* (TAVCV)

Introduction

Rhabdoviridae is a versatile family of viruses that cause diseases in a wide variety of organisms including eukaryotes, marine-life, humans and economically important animals and plants (Hogenhout, Redinbaugh, & Ammar, 2003). Although virulence ranges across many life forms, a special case is presented with the infection of plants in that all plants are immobile. Once inside the host, the virus is essentially confined to that one plant and location unless actively transferred. Insects, therefore, perform an important role in the transmission and continued existence of most rhabdoviruses.

Leafhoppers, planthoppers, aphids and lacebugs so far have been discovered to transmit plant infecting rhabdoviruses (Nault, 1997) in a persistent propagative manner (Sylvester & Richardson, 1992; Ammar, 1994; Howerth, Mead, & Stallknecht, 2002). Because rhabdoviruses replicate once inside their vectors in order to be transmitted, it could be thought that two natural hosts exist for each of these rhabdoviruses; the vertebrate infected and insect vector or the plant infected and arthropod vector (Hogenhout et al., 2003).

A few rhabdoviruses that affect plants can also be transmitted mechanically. *Maize mosaic virus* (MMV), genetically the closest relative of TaVCV, showed up in maize plants after artificial inoculation of kernels with extracts prepared from infected leaves and roots (Louie, 1995). Another close relative, *Eggplant mottled dwarf virus* (EMDV) was found transmissible with direct sap inoculation of potato plants using leaf extracts from three systemically infected

Nicotiana species (Danesh, 1989). Mechanical transmissibility of EMDV, however, has been known since the 1970s (Martelli & Rana, 1970). *Sonchus yellow net virus* (SYNV) is mechanically transmissible to *Nicotiana glutinosa* and *N. clevelandii* individually as well as their hybrid (*N. clevelandii* × *N. glutinosa*), sowthistle (genus *Sonchus*, family *Asteraceae*), *Bidens pilosa* (family *Asteraceae*), *Zinnia elegans* (family *Asteraceae*) and lettuce (*Lactuca sativa*, family *Asteraceae*) (Christie, Christie, & Edwardson, 1974).

Outside of the *Nucleorhabdovirus* genus, *Wheat American striate mosaic virus* (WASMV; previously *Wheat striate mosaic virus* - WSMV), a *Cytorhabdovirus*, was successfully introduced into its leafhopper vector by injection with low speed clarification extract or high speed (105,000 *g*) pellet suspension; the leafhoppers were then able to transmit the virus to wheat plants (Lee, 1963). In Brazil, *Coffee ringspot virus* (CoRSV), a *Dichoravirus*, was transmitted to some herbaceous hosts using infected sap (Chagas, July, & Alba, 1981). The ability of TaVCV to be transmitted mechanically or via seeds is currently unknown.

While rearing taro plants for transmission studies, taro planthoppers, two species of aphids and later one species of mealybug were found to naturally appear on some of the plants. These insect colonies were maintained and used for TaVCV tests and a transmission trial (planthoppers only). It is important to note that members of the genus *Nucleorhabdovirus* have so far been found to be transmitted only by planthoppers, leafhoppers and aphids (as discussed in section 4.5.3 of chapter 1). The taro planthopper *Tarophagus proserpina* has long been known to harbor virus-like particles (Dabek & Plumb, 1975) and is already associated with the transmission of the *Cytorhabdovirus Colocasia bobone disease virus* (CBDV) (Gollifer et al., 1977). The insect responsible for transmitting TaVCV in nature remains to be identified. This chapter discusses efforts made towards determining the vector for TaVCV.

Materials and Methods

TaVCV source plants and insect samples

Plants displaying typical taro vein chlorosis disease symptoms and taro planthoppers feeding on these were collected from the University of Hawaii's Waimanalo Research Station, Waimanalo, HI. Early into the study, two asymptomatic plants that were RT-PCR negative for TaVCV using primer set Pol2A1/Pol2A2 (Revill et al., 2005) were received from the University of Hawaii's Kona Research Station, Kainaliu, HI. Taro planthoppers that naturally emerged on these plants were maintained. Asymptomatic but RT-PCR positive taro plants (using primer set DCGF5/DCGR5) from Waimanalo as well as a commercially purchased asymptomatic but RT-PCR and IC-RT-PCR positive taro plant (using primer set DCGF5/DCGR5) were also used to sustain taro planthopper, aphid and mealybug colonies. All the source plants and insects feeding on them were kept inside insect cages 2.5' x 1.5' x 2' in dimension, made using insect proof greenhouse shade cloth.

Testing insect samples

Taro planthopper samples were collected in November of 2015 and tested in groups of 1 (10 samples), 5 (5 samples) and 10 (5 samples). Aphids and mealybugs were tested in 3 successive collections – February, April and May of 2016; all in groups of 10. Total nucleic acid was extracted using the CTAB method: first, the insects were ground in 120 µL of CTAB buffer [2% Cetyltrimethyl ammonium bromide (CTAB) (w/v) 20 g, 100mM Tris (pH 8.0, 1M) 100 mL, 20mM EDTA (pH 8.0, 0.5 M) 40 mL, 1.4M NaCl 81.8 g, 1% PVP (Mr40,000) 5 g, ddH_2O to 1 L]. Then, 100 µL chloroform was added and the mixture was vortexed for 2 minutes followed by centrifugation at 5000 *g* for 2 minutes. The supernatant was transferred to a fresh 1.5 mL microfuge tube and 1 volume of isopropanol was added. After gentle swirling and mixing, the

samples were allowed to stand for 5 minutes then spun at maximum speed for 20 minutes followed by removal of the supernatant. The resulting pellet was re-suspended in 20 µL sterile water. Reverse transcription (RT)-PCR was performed using primer set DCGF5/DCGR5 as described in CHAPTER 3.

Initial transmission trial

First, samples were collected from symptomatic, RT-PCR positive (primer set Pol2A1/ Pol2A2) taro plants from Waimanalo and planthoppers feeding on it as well as asymptomatic, RT-PCR negative (primer set Pol2A1/Pol2A2) taro plant from Kainaliu and its planthoppers. Samples were stored at -80°C. Tissue cultured taro plants of the varieties Lehua ‘Ele’ele, Lauloa ‘Ele’ele Oma’o and Maea at the 2-3 leaf stage obtained courtesy of Lyon Arboretum and maintained by Ms. Miriam Long were planted into soil-less medium. Leaf samples were collected from each of these plants and stored at -80°C before the plants were used in the trial.

In June 2015, five planthoppers from the symptomatic, RT-PCR positive (primer set Pol2A1/Pol2A2) taro plants were introduced onto each of five of the tissue cultured plants (labelled P1 – P5) now in soil-less medium. Similarly, five planthoppers each from the asymptomatic RT-PCR negative (primer set Pol2A1/Pol2A2) plant were placed onto five other tissue cultured plants (labelled -P1 – -P5) in soil-less medium. Each of these 10 plants were put into insect cages specially made using clear wide-mouth polyethylene plastic jars and insect proof greenhouse shade cloth, closed-off carefully and kept in a growth chamber conditioned at a temperature of 20°C, mild humidity and 16 hours light/8 hour dark cycles. The planthoppers were removed after 4 days (96 hours) of feeding while the plants were allowed to grow for a further 10 weeks (70 days).

Leaf samples from four plants (P1, P2, P4, P5; P3 was dead) that received planthoppers from the symptomatic, RT-PCR positive taro plant and one plant (-P4) that received planthoppers from the asymptomatic, RT-PCR negative taro plant were collected for analysis. RNA extraction and RT-PCR were performed as previously described, first with primer set Pol2A1/Pol2A2 and then with primer sets DCGF1/DCGR1, DCGF2/DCGR2, DCGF3/DCGR3, DCGF4/DCGR4, DCGF5/DCG R5 and DCGF6/DCGR6 as they became available.

Simple virus localization assay

With the knowledge of plant *Nucleorhabdovirus* behavior, that is, replication and movement to different organs once inside the insect vector (Nault & Ammar, 1989; Ammar, 1994; Nault, 1997; Hogenhout et al., 2003; Ammar & Hogenhout, 2008), a dissection experiment was designed to investigate TaVCV presence in different organs of aphids and planthoppers obtained from symptomatic, RT-PCR positive (with primer set DCGF5/DCGR5) taro plants. Dissection was conducted a total of four times: once in September 2016 and 3 times in October 2016. Taro planthoppers and aphids feeding on a RT-PCR negative (with primer set DCGF5/DCGR5) taro plant were included as control in the second and third dissection. A basic dissection kit was used to separate aphid head, guts, the remaining body and embryos as well as planthopper head, salivary glands, guts and the remaining body with the aid of a compound microscope fixed with photo and video recording capabilities. Care was taken to disinfect equipment with 70% ethanol between and with-in samples. A set of 5 – 6 insects were dissected per sample per test. Dissected parts were immediately placed into buffer RA1, the lysis buffer in the Macherey-Nagel Nucleospin® RNA II kit and kept on ice. RNA extraction and RT-PCR (using primer set DCGF5/DCGR5) were then conducted as previously described. Whole aphid

and planthopper samples were included in subsequent tests following results obtained in the first dissection experiment.

Results

TaVCV in insect samples

Taro planthoppers, aphids and later mealybugs naturally appeared on TaVCV infected taro plants kept inside insect cages in a growth room or the green house. Their eggs and/or nymphal stages were presumably in the accompanying soil or between the leaf petioles when the plants were brought from the field and planted in the greenhouse. The planthoppers were tested by RT-PCR using primer set DCGF5/DCGR5 in groups of 1, 5 and 10 while aphids and mealybugs were tested similarly but in groups of 10 over three successive collections. 95% of taro planthopper, 65% aphid and 68% mealybug samples were positive for TaVCV. Interestingly, none of the samples in the second aphid and mealybug collection were positive. (Table 4.1).

Initial transmission trial

Four tissue-cultured taro plants (P1, P2, P4, P5) that received planthoppers from the symptomatic, RT-PCR positive (primer set Pol2A1/Pol2A2) taro plant and one tissue-cultured plant (-P4) that received planthoppers from the asymptomatic, RT-PCR negative (primer set Pol2A1/ Pol2A2) taro plant were tested for TaVCV with primer pair Pol2A1/Pol2A2. TaVCV was not detected in any of these 5 plants. However, all of the six new primer sets detected TaVCV in all five of the tissue cultured plants (Table 4.2).

Table 4.1. TaVCV test on insect samples collected from TaVCV infected taro plants. n(T) = number of samples tested, n(P) = number of samples TaVCV positive by RT-PCR.

Insect	Collection #	Group of	n(T)	n(P)
Planthoppers	1	1	10	9
		5	5	5
		10	5	5
Total			**20**	**19**
Aphids	1	10	17	17
	2	10	18	0
	3	10	20	19
Total			**55**	**36**
Mealybugs	1	10	8	8
	2	10	8	0
	3	10	9	9
Total			**25**	**17**

Table 4.2. TaVCV test of 5 tissue cultured plants used for the initial transmission trial. Pol2A1/ Pol2A2 failed to detect TaVCV in these plants while all the 6 new primer sets did.

Plant	Primer Set							
	DCGF1/ DCGR1	**DCGF2/ DCGR2**	**DCGF3/ DCGR3**	**DCGF4/ DCGR4**	**DCGF5/ DCGR5**	**DCGF6/ DCGR6**	**POL 2A1/2**	**NC**
P1	+	+	+	+	+	+	**-**	-
P2	+	+	+	+	+	+	**-**	-
P4	+	+	+	+	+	+	**-**	-
P5	+	+	+	+	+	+	**-**	-
-P4	**+**	**+**	**+**	**+**	**+**	**+**	**-**	-

To determine if the asymptomatic taro plants and planthoppers from the Kona Research Station (RT-PCR negative using primer set Pol2A1/Pol2A2) were TaVCV positive to begin with, they were tested again with primer set Pol 2A1/Pol2A2, TaVCV1/TaVCV2 and additionally with DCGF5/DCGR5 and found to be positive; however, only with primer set DCGF5/DCGR5 (Fig. 4.1). The leaf samples from the 10 tissue-cultured plants taken at the beginning of the trial and stored at -80°C were tested by RT-PCR using primer set DCGF5/DCGR5 and also found to be TaVCV positive (Fig. 4.2).

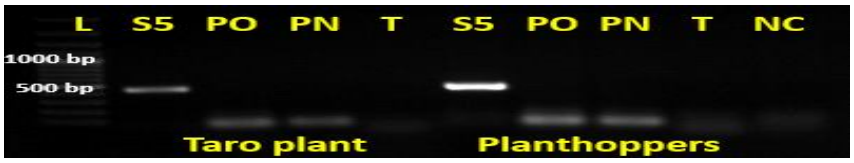


Fig 4.1. The asymptomatic taro plant from Kona Research Station and the planthoppers feeding on it, previously TaVCV negative when tested with primer set Pol2A1/Pol2A2, show up as TaVCV positive when tested with primer pair DCGF5/DCGR5. Primer set TaVCV1/TaVCV2 failed to detect TaVCV in these samples. S5 = DCGF5/DCGR5, PO = Pol2A1/Pol2A2 old tube, PN = Pol2A1/Pol2A2 new tube, T = TaVCV1/TaVCV2, NC = Negative control, L = Ladder.

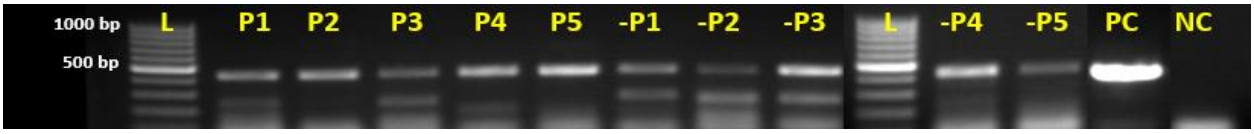


Fig 4.2. the 10 tissue cultured plants used for the transmission trial turn out to be TaVCV positive; tested with primer set DCGF5/DCGR5. P1 – P5 = received planthoppers from the symptomatic, RT-PCR positive (primer set Pol2A1/Pol2A2) taro plant. -P1 – -P5 = received planthoppers from the asymptomatic, RT-PCR negative (primer set Pol2A1/ Pol2A2) taro plant. PC = positive control, NC = negative control, L = Ladder.

TaVCV localization in taro planthopper

A simple dissection experiment was conducted to separate major body parts of taro planthoppers and aphids to test for TaVCV. These two insects were of interest for two reasons: one, taro planthoppers and aphids were most commonly noticed feeding on taro leaves during field visits. Secondly, planthoppers and aphids are known vectors in the *Rhabdoviridae* family. In the first dissection experiment, TaVCV was detected by RT-PCR in taro planthopper head region, salivary glands, guts and remainder of the body but not in aphid head (including salivary glands), guts, remaining body and embryo retrieved from anatomized females (Fig. 4.3).

TaVCV was not detected in either taro planthopper or aphid samples in the second and third dissections. These insects were collected from the same RT-PCR TaVCV positive plants as the first dissection. In these two tests TaVCV was not detected in both taro planthopper and aphid samples collected from RT-PCR TaVCV negative taro plants. TaVCV was once again detected from whole taro planthoppers, their head region, salivary glands, guts and remainder of the body as well as in aphids tested in groups of 5 after the fourth dissection (Table 4.3).

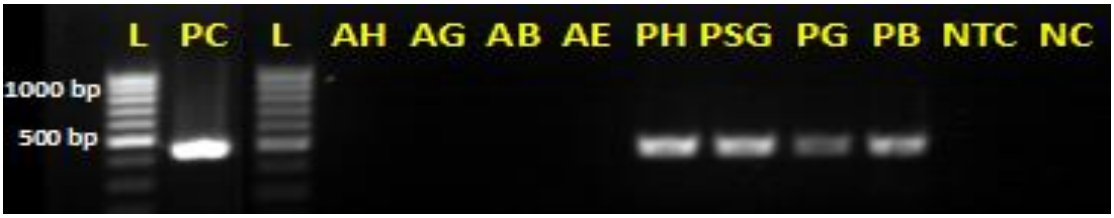


Fig 4.3. RT-PCR detection of TaVCV in taro planthopper body parts but not in aphid body parts. AH = aphid heads, AG = aphid guts, AB = aphid bodies, AE = aphid embryos, PH = planthopper head, PSG = planthopper salivary glands, PG = planthopper guts, PB = planthopper body, L = ladder, PC = positive control, NTC = non-template control, NC = water control.

Table 4.3. TaVCV test of whole taro planthoppers and aphids and their body parts after dissection. TaVCV was detected from taro planthoppers and their body parts in the first and fourth dissection and only in whole aphids in groups of 5 in the fourth dissection.

NA = not included

Insect or Insect Part	**1st Dissection**	**2nd Dissection**	**3rd Dissection**	**4th Dissection**
Taro planthoppers (P)	NA	-	-	+++
P Head Region	+++	-	-	++
P Salivary Glands	+++	-	-	++
P Guts	++	-	-	++
P Bodies	++	-	-	++
Aphids (A)	NA	-	-	+
A Head Region	-	-	-	NA
A Guts	-	-	-	NA
A Bodies	-	-	-	NA
A Embryos	-	-	-	NA
Positive Control	+++	+++	+++	+++
Non-template Control	-	-	-	-
Water Control	-	-	-	-

+++ = very strong band, ++ = strong band, + = weak band

Discussion

Taro planthoppers and aphids were the two most common insect pests noted on taro during field visits and were also the first to infest taro plants being grown for the purpose of this research. Mealybugs were also maintained, however, their numbers declined and ultimately all died towards the later stages of the project. The capacity of an insect to successfully transmit a plant virus is somewhat determined or conversely constrained by host range and its own feeding habits (Fereres & Moreno, 2009), therefore, insects obtaining nourishment from the same host plant do not necessarily vector the same virus(es) (Hogenhout et al., 2003). This specificity in virus-vector relationship is clearly demonstrated by 2 maize feeding insects: the leafhopper *Graminella nigrifons* and planthopper *Peregrinus maidis*. The former transmits *Maize fine streak virus* (MFSV) but not *Maize mosaic virus* (MMV) while the latter vectors MMV but not MFSV (Redinbaugh et al., 2002). Both MMV and MFSV are nucleorhabdoviruses in the *Rhabdoviridae* family.

In similar fashion, it is known that planthoppers and aphids transmit members of the genus *Nucleorhabdovirus*, but it does not necessarily mean that feeding on TaVCV infected taro enables both insects to transmit TaVCV. Mealybugs, so far, have not been associated with the transmission of any plant infecting rhabdovirus. TaVCV was detected by RT-PCR in whole taro planthoppers, aphids and mealybugs feeding on TaVCV infected plants (Table 4.1). These tests showed that all of the insects ingest the virus, however, a positive RT-PCR test on the insect samples alone cannot determine if infectious units were being detected and certainly provides no information as to whether these arthropods can inoculate another taro plant with TaVCV. Because all of the insects under study were plant sap feeders, virions could have been uptaken together with the cell contents while feeding. In fact, humans also have tested positive through

feces analysis for many plant viruses ingested via food products (Zhang et al., 2005) and this does not imply that humans are vectors of plant viruses.

An intriguing observation was made over successive collection and testing of aphid and mealybug samples maintained on RT-PCR TaVCV positive taro plants. None of the 18 aphid and 8 mealybug samples in the second batch were RT-PCR positive for TaVCV, collected 7 weeks after the first batch in which all the 17 aphid and 8 mealybug samples were TaVCV positive. In the third batch, collected 4.5 weeks after the second batch, all the 20 aphid and 9 mealybug samples were positive for TaVCV once again (Table 4.1). One possible explanation for this is that the plants may have 'suppressed' virus levels enough for it not to be picked up by new insect progenies hence not be detected in the second collection and then later TaVCV 'resurged' to be detected in the third collection. The ability of virus infected plants to 'reverse' infection has been shown in the root crop – cassava (*Manihot esculenta*) graft inoculated with *Ugandan cassava brown streak virus* (UCBSV) and *Cassava brown streak virus* (CBSV), both members of the genus *Ipomovirus* in the *Potyviridae* family. A significant proportion of short 10 cm cuttings taken from upper stems of diseased plants produced virus-free plants in the next generation at much higher percentages compared to middle and lower parts (Mohammed, Ghosh, & Maruthi, 2016). While taro and cassava are plants in two completely different groups and each possesses a unique set of physiological characteristics, that is, the former is a monocot and the latter a dicot, host-virus interactions are still of interest.

A similar case as above was noticed in taro planthopper and aphid dissection tests. TaVCV was detected by RT-PCR in taro planthopper body parts but not in aphid body parts or embryo in the first dissection. TaVCV was not detected in both taro planthopper and aphid samples in the second and third dissection 2.5 and 2 weeks apart, respectively. Then, TaVCV

was once again detected in whole aphids (group of 5) and taro planthopper body parts in the fourth dissection, however, these samples were sourced from another TaVCV RT-PCR and IC-RT-PCR positive plant. Taro planthopper population drastically increased on WPT-GR in the insect cage (which normally does not happen in the field) after the first dissection while aphids heavily infested WAK5 and WAK6 subjecting the plants to stress and affecting normal growth and development. Due to enclosure, the plants may have been under heat and resulting water-stress as well. It could be the case that TaVCV did not manifest itself well in the stressed plants and virus levels receded, therefore, new generations of taro planthoppers and aphids probably did not pick up the virus efficiently. Willful insect feeding may also have altered for these plants. Hence, no TaVCV was detected in these insects in the second and third dissection. Indeed, water stress and a temperature of 30°C together led to a lowered disease incidence caused by *Barley yellow streak mosaic virus* (BaYSMV; genus *Cytorhabdovirus*, family *Rhabdoviridae*) transmitted by the brown wheat mite *Petrobia latens* (Smidansky, 1996). Likewise, reduced virus incidence and transmission rate were noted in the soybean aphid and aphid-transmitted, *Soybean mosaic virus* (SMV; genus *Potyvirus*, family *Potyviridae*) system when soybean plants were under drought-stress (Nachappa et al., 2016). In these plants, petiole exudates contained high levels of total free amino acids including asparagine and valine that are important for aphid performance. Aphids seemed not to benefit from the enhanced phloem sap quality; their numbers remained low on drought-stressed plants.

Like the insect samples, some taro plants that were kept to rear these insects also showed variable results over subsequent tests. WAK5, WAK6, WPT-GR and taro planthoppers feeding on this plant (PH-GR) which were all RT-PCR positive in initial tests later tested negative for TaVCV by IC-RT-PCR. For confirmation, WPT-GR was assayed by RT-PCR after IC-RT-PCR

and the test returned negative for TaVCV. This plant was highly symptomatic for TaVCV when first brought from Waimanalo but new leaves did not show symptoms after the symptomatic leaves senesced. This observation has been reported previously (Carmichael et al., 2008; Harding, n.d.). Cranberry plants that become infected with *Tobacco streak virus* (TSV; genus *Ilarvirus*, family *Bromoviridae*) and produce scarred, symptomatic, TSV-positive fruits in one year have been studied to output non-scarred, asymptomatic, TSV-positive fruit in succeeding growing seasons (Wells-Hansen & McManus, 2016). While taro leaves and cranberry fruits are different organs affected by their respective viruses, the 'recovery from symptoms' phenomena can still be correlated. Overall, TaVCV tests on the 3 insect samples, the insect dissection assays and results from repeated testing of some plant samples over a period of time indicate a lot of dynamics are at interplay between taro plants and TaVCV after infection. Temporal virus distribution as well as within plant distribution of TaVCV is not well understood nor studied and are important areas of investigation for the future.

In the insect dissection assays, TaVCV was detected by RT-PCR in the taro planthopper head region, salivary glands, guts and remaining body but not in the aphid head region, guts, body and embryo in the first dissection. In the fourth dissection, TaVCV was detected in whole aphids (groups of 5) and once again in whole taro planthoppers, head region, salivary glands, guts and remaining body. Because rhabdoviruses are transmitted in a persistent propagative manner and replicate inside their vectors (Jackson, Francki, & Zuidema, 1987; Nault & Ammar, 1989; Sylvester & Richardson, 1992; Jackson et al., 2005; Redinbaugh & Hogenhout, 2005; Ammar & Hogenhout, 2008; Mann & Dietzgen, 2014), their detection in different tissues of the taro planthopper may well suggest that taro planthopper is the vector for TaVCV. *Sowthistle yellow vein virus* (SYVV), another *Nucleorhabdovirus* is found in various tissues of non-vector

aphids but not in the salivary glands (Sylvester & Richardson, 1992), therefore, the detection of TaVCV in salivary glands of the taro planthopper supports the case.

The taro planthopper, *Tarophagus proserpina*, is already known to vector the *Colocasia bobone disease virus* (CBDV), however, it is not uncommon for one insect to transmit multiple viruses. For example, the silverleaf whitefly (also known as the sweetpotato whitefly; *Bemisia tabaci*) vectors the *Sweet potato leaf curl virus* (SPLCV; genus *Begomovirus*, family *Geminiviridae*), *Ipomoea leaf curl virus* (ILCV; genus *Begomovirus*, family *Geminiviridae*) and the *Sweet potato mild mottle virus* (SPMMV; genus Ipomovirus, family *Potyviridae*) for the same host – sweet potato (*Ipomea batatus*) (Valverde, Sim, & Lotrakul, 2004). At this point, the combined results from RT-PCR test on single whole insects, in groups and dissected parts point towards the taro planthopper, *Tarophagus proserpina*, as the insect that transmits TaVCV.

CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

The *Taro vein chlorosis virus* (TaVCV), although a recent discovery in Hawaii (Long et al., 2014), is fast becoming a concern for the entire state (Cruz, 2016). The interveinal chlorosis (netted yellow striping between the veins), downward bending of the leaves and necrosis at the leaf margins greatly reduces the commercial value of the leaves and presumably the photosynthetic ability of the plant. Some varieties develop streaking in their petioles, while some also become stunted due to infection. Plant death may occur in severe cases. This was especially observed in plants with simultaneous heavy infestation by sap feeding insects such as aphids and planthoppers. The direct effect of TaVCV infection on taro corm yield has not been studied and is an important area of investigation.

Comparison of the nucleotide and amino acid sequences as well as phylogenetic analyses of the partial RdRp gene revealed very low levels of genetic diversity at both nucleotide and translated protein levels for TaVCV within and between Hawaiian and Palauan isolates. When the same analyses were expanded to include the partial RdRp sequence of a Fijian isolate obtained from GenBank (accession AY674964), LALIGN sequence variability between the Hawaiian/Palauan isolates versus the Fijian isolate escalated to 22% at the nucleotide level and 8.7% at the protein level. An earlier study reported such high levels of diversity within Pacific TaVCV isolates (Revill et al., 2005a), however, their sequence data was not available for inclusion in this study. The high variance noted between Hawaiian/Palauan sequences and that of the Fijian isolate suggest more than one TaVCV strain may exist. Full-length genome sequencing of Pacific TaVCV isolates will help to resolve this knowledge gap. Current phylogenetic evaluation tends towards a general grouping of TaVCV isolates based on geographic location.

Survey and inclusion of sequence data from more countries in the Pacific will add or reduce support to this observation as well as help identify new areas where TaVCV occurs.

Six new primer sets were designed from TaVCV sequence data targeting different regions of the RdRp gene and are now available for use in future surveys or surveillance and monitoring of TaVCV. Primer pair DCGF5/DCGR5 is by far the most sensitive and efficient primer available for TaVCV detection by reverse transcription-polymerase chain reaction (RT-PCR). This set helped detect more TaVCV positive samples from Hawaiian and Palauan isolates compared to primer sets Pol2A1/Pol2A2 and TaVCV1/TaVCV2 created by Revill et al. (2005a). The risks of obtaining false negatives and not detecting TaVCV in low-titer samples is greatly reduced with the availability of the robust RT-PCR assay established using primer set DCGF5/ DCGR5.

Significant progress was made towards developing a high-throughput serological detection assay for TaVCV. Three polyclonal antibodies were raised against short, 15 amino acid long peptides artificially synthesized from the parent nucleocapsid gene amplified with primer set Cap2A/Cap2B (Revill et al., 2005a). An indirect-enzyme linked immunosorbent assay (ID-ELISA) protocol was established for TaVCV as part of this project. The method showed promise with the anti-TaVCV-N-1 antisera, however, efforts were halted when non-specific reactions against RT-PCR negative and non-host plants were obtained. Instead, an immunocapture-RT-PCR (IC-RT-PCR) assay was developed. The IC step traps virus particles while the RT-PCR effectively distinguishes TaVCV from non-target sequences. The IC-RT-PCR is currently a two-step system, hence, establishment of a one-step IC-RT-PCR protocol for TaVCV will help make the technique efficient as well as highly throughput and less time consuming than RT-PCR.

These detection methods are good tools for plant quarantine and germplasm centers distributing taro.

One of the major challenges for this project was obtaining 'true' TaVCV free plants for transmission trials. This was further complicated when tissue-cultured taro from Lyon Arboretum and taro plants from University of Hawaii's Kona Research Station, Kainaliu, HI used for a preliminary transmission were negative for TaVCV using primer pair Pol2A1/Pol2A2, but subsequently found to be positive with primer set DCGF5/DCGR5. Members of the genus *Nucleorhabdovirus* are transmitted persistently in a propagative and circulative manner. This has been studied extensively in the *Maize mosaic virus* (MMV; genus *Nucleorhabdovirus*, family *Rhabdoviridae*) and its planthopper (*Peregrinus maidis*) vector system (Ammar & Hogenhout, 2008). Genetically, MMV is the closest relative of TaVCV, hence a similar mode of transmission is assumed for TaVCV. A simple virus localization test was conducted and TaVCV was repeatedly detected in whole taro planthoppers (*Tarophagus proserpina*; family Delphacidae), their head region, salivary glands, guts and remaining body while only being detected in whole aphids but not in their head region, guts, remaining body and embryo. Because nucleorhabdoviruses replicate inside their vectors and localize into the salivary glands for transmission, the virus localization assay provided strong evidence that the taro planthopper is the vector insect for TaVCV. This knowledge is vital for devising future management and control options for the taro vein chlorosis disease.

Two special cases of variable results obtained from RT-PCR tests; firstly, from whole aphid and mealybug samples collected from the same TaVCV infected plants over several months and secondly, from 3 previously RT-PCR positive or highly symptomatic taro plants maintained at the greenhouse or inside an insect cage in a growth room at ambient conditions

indicate a lot of dynamics are at interplay between taro plants and TaVCV after infection. Temporal and within plant distribution of TaVCV and the effect of environmental conditions, especially temperature on taro plant health and TaVCV fitness is not well understood nor studied and are important areas of research for the future.

APPENDICES: LIST OF NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES

Appendix A: List of nucleotide sequences

1. The list of partial L (RdRp) nucleotide sequences used for genetic diversity studies:

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>TM713*
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*These two isolates from Maui were omitted from phylogenetic analyses due to anomalies in their data

Molokai (from GenBank; accession KF921086)
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>TFiji
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>TM48
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>TM72
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TCAGGAGCAGCTGAGGATGCGGCGACAAAGGCTAAAGCCATATCCTTCCTATGCTG
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>TM723
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TCAGGAGCAGCTGAGGATGCGGCGACAAAGGCTAAAGCCATATCCTTCCTATGCTG
CGCACTGATACGATTATCCGTCAAGGAGCCCAGCCATATAATGACCGCCATAACCA
GCATCAGACAGCGGTTTGGCTCTCTTTACGGTTTATCCTCAGCTACATTGAATGCTAT
TACATTCACCCGCCAGCAGCTATCGAGGATTAAACAGGGGATAGAAACCTATTCTGT
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AATGCACATCTATAAGATGATATTGGCCCTGCTCACTGAATGGTCGACTGTCCCAAT
TGGACTACTCCTAACTTGGATAAGGAACCCAAAATCAGCTCTGGCAGTCACTGAAAT
TAAGAATATAATAACAAATTTTGACAAGGCAGGAGTAGATAAGTCCTGGAAGTACT
CTAGGATGATAGACAACACCTTCTTTTTGAACATTTCATCAAGGAGGAATGTTTATA
TGTGTGCATTGTTGGCCTCATTAAACAAGAGGCATGTCCCTCAAGGAGTGGGAGACT
ATGCCGATCCTCGGAACATTGCTGTGATCAAGGCAATGGATGCAGCAGTGAAGAAC
CAGGTGGCAATTGATGTCACCTTGGTGGAACGCATTTATGAGAAATACTTAATTTCT
GCAGGTTCAACCGATGCCGGCACCGCATACACCCTCTCTCGAGGCACCAAGAGGCC
CAATCCAGCCGCCACTGAACCCTCCACAA

Oahu
>TO68*
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ATACCATTCCCTCAGACTGGTCCAGCAGCATCAGCACTGACGGGGTGGACACAACA
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CGCACTGATCCGATTATCCGTCAAGGAGCCCAGCCATATAATGACCGCCATAACCA
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TGCAAGAGGGACCATCTTTTACTACGTGAGATATGCAGACACTACGTATACGAGCTC
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TAAGAATATAATAACAAATTTTGACAAGGCATGAGTAGATAAGTCCTGGAAGTACT
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TGTGTGCATTGTTGGCCTCATTAAACAAGAGGCATGTCCCTCAATGAGTGGGAGACT
ATGCCGATCCTCGGAACATTGCTGTGATCAAGGCAATGGATGCAGCAGTGAAGAAC
CAGGTGGCAATTGATGTCACCTTGGTGGAAGGCATTTATGAGAAATACTTAATTTCT
GCAGGTTCAACCGATGCCGGCACCGCATACACCCTCTCTCGAGGCACCAAGAGGCC
CAATCCAGCCGCCACTGAACCCTCCACAA

*This isolate from Oahu, the only sample for which the N gene was sequenced has anomalies in its data. There is a sudden stop codon at position 231 when this nucleotide sequence is translated.

Molokai (from GenBank; accession KF921085)
>TMol
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TCAGGAGCAGCTGAGGATGCGGCGACAAAGGCTAAAGCCATATCCTTCCTATGCTG
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TGACAAATCGTATGGTGTCTGCCGATTCCTTCTATTCCAGCACCTTGAGTTAGAAGG
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TGTGTGCATTGTTGGCCTCATTAAACAAGAGGCATGTCCCTCAAGGAGTGGGAGACT
ATGCCGATCCTCGGAACATTGCTGTGATCAAGGCAATGGATGCAGCAGTGAAGAAC
CAGGTGGCAATTGATGTCACCTTGGTGGAACGCATTTATGAGAAATACTTAATTTCT
GCAGGTTCAACCGATGCCGGCACCGCATACACCCTCTCTCGAGGCACCAAGAGGCC
CAATCCAGCCGCCACTGAACCCTCCACAA

Appendix B: List of deduced amino acid sequences

1. The list of partial L (RdRp) amino acid sequences used for genetic diversity studies:

Oahu
>TO64
MPNLFDITHDLFRESVIYLCSGEGNLRGDPTFGVAPDGVWSWTGDESGKEGLRQKGWTI
LTVVTIMLIAKRHHVDVSLMGGGDNQVLGITIGGMVRDSVGELTQDSCRLAQSTINRFT
QDLIKTFGDLGLPLKASETWVSDSLFMYNKHMFHKGMPLRSPLKAISRIFPLANDSIMTL
DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG
VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
EMRRYIVDPGLASV

>TO68
MPNLFDITHDLFRESVIYLCSGEGNLRGDPTFGVAPDGVWSWTGDESGKEGLRQKGWTI
LTVVTIMLIAKRHHVDVSLMGGGDNQVLGITIGGMVRDSVGELTQDSCRLAQSTIKRFT
QDLIKTFGDLGLPLKASETWVSDSLFMYNKHMFHKGMPLRSPLKAISRIFPLANDSIMTL
DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG
VVTRAGRRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
EMRRYIVDPGLASV

Hawaii
>TH94
MPNLFDITHDLFRESVIYLCSGEGNLRGDPTFGVAPDGVWSWTGDESGKEGLRQKGWTI
LTVVTIMLIAKRHHVDVSLMGGGDNQVLSITIGGMVRDSVGELTQDSCRLAQSTIKRFT
QDLIKTFGDLGPPLKASETWVSDSLFMYNKHMFHKGMPLRSPLKAISRIFPLANDSIMTL
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VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGNLHLPDIVMRGFPDPLTSHLSFI
AEMRRYIVDPGLASV

>TH98
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EMRRYIVDPGLASV

>TH911
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DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG
VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
EMRWYIVDPGLASV

>TH923
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DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG
VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
EMRRYIVDPRLAS

Kauai
>TK21
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QDLIKTFGDLGLPLKASETWVSDSLFMYNKHMFHKGMPLRSPLKAISRIFPLANDSIMTL
DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG
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EMRRYIVDPGLASV

>TK22
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>TK24
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DNMVNNISSGVKAACMKERHGIPLVFVKTMAYRRVAELSLVLHPLTTCFKKPELPDHG

VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
EMRRYIVDPGLASV

>TK71
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EMRRYIVDPGLASV

>TK72
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TKMRRYIVDPGLASV

>TK73
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QDLIKTFGDLGLPLKASETWVSDSLFMYNKHMFHKGMPLRSPLKAISRIFPLANDSIMTL
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VVTRAGKRVKISVTSKNLRQYFGLCMLGSSTMGHPGTLHLPDIVMRGFPDPLTSHLSFIA
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>TK74
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*These two isolates from Maui were omitted from phylogenetic analyses due to anomalies in their data

Fiji (complete L (RdRp) protein sequence from GenBank; accession YP_224083)
>TFiji
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2. The list of partial N (Nucleocapsid) amino acid sequences:

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MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM72
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM75
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM78
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGACRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI

KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM711
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTFVGRIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM713
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TM723
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKAGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYA
DPRNIAVIKAMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAA
TEPST

>TO68*
MTAITSIRQRFGSLYGLSSATLNAITFTRQQLSRIKQGIETYSVARGTIFYYVRYADTTY
TSSDKSYGVCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVTEI
KNIITNFDKA

*This isolate from Oahu, the only sample for which the N gene was sequenced has anomalies in its data. There is a sudden stop codon at position 231.

Fiji (complete N (Nucleocapsid) protein sequence from GenBank; accession YP_224078)
>TFiji
MSYINIPDDVVSKYSDDLKTFTQKAGEIPSSKSLIPQTAYTIAALKTKLKFWEVTAKDDP
TIASDWAGVCTAITAGTFSATNLKTVCELAFNLRKPHETGNVFIHTVPSDWTSSISTDSV
DTTPIPATESDATLSTVSAAVQSGAAEDAATKAKAISFLCCALIRLSVKEPSHIMTAITSIR
QRFGSLYGLASATLNAITFTRQQLSRIKQGIETYSLARGTIFYYVRYADTTYGSSDKSYG
VCRFLLFQHLELEGMHIYKMILALLTEWSTVPIGLLLTWIRNPKSALAVVEIKNIITNFDK
AGVDKSWKYSRMIDNTFFLNISSRRNVYMCALLASLNKRHVPQGVGDYADPRNIAVIK
AMDAAVKNQVAIDVTLVERIYEKYLISAGSTDAGTAYTLSRGTKRPNPAVFMSHQQAE
GHPTKKRTWKSMVPPPPTLQLEDQQQRRLSPELCEDCFSELLRGKHHRILPPPVIPSSSSE
VLFSKSRIMEISGGCSFDPIGF

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